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Study of The Role of Macrophage Activation and Macrophage Derived Cytotoxic Factors in Early Embryo Loss

by

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A thesis submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

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Submitted on February 14th

Abstract

Using murine models of spontaneous and induced embryo resorption, we have investigated the role of macrophages in the mechanism of early embryo loss. The results showed that macrophage derived nitric oxide was associated with embryo resorption, and that decidual macrophages could be triggered by lipopolysaccharide (LPS) to produce nitric oxide, indicating that the decidual mononuclear cells were primed *in situ*. Using double immunostaining, we have shown that macrophages were the cellular source of the inducible nitric oxide production. We further showed that embryo abortion can be significantly decreased by inhibiting the production of nitric oxide *in vivo*. The results presented strongly suggested a role for nitric oxide as an effector molecule in mediating early embryo loss and showed that the *in situ* activation of decidual macrophages was an early event preceding spontaneous abortion.

It is known that interferon-γ (IFN-γ) is the major cytokine responsible for the priming of macrophages and that LPS can trigger primed macrophages to produce nitric oxide. Therefore, the observation that exogenous LPS induced embryo abortion in most strains of pregnant mice suggested that the decidual macrophages have been

previously primed *in situ*. To investigate the role of IFN-γ as a potential priming signal for decidual macrophage activation, we studied the effect of the depletion of IFN–γ on LPS induced pregnancy loss. The results showed that IFN–γ deficient mice were more resistant to LPS induced abortion than control mice. This suggested that IFN–γ was essential for the priming of decidual macrophages and that decidual macrophages from IFN-g deficient mice could not be activated when exposed to LPS both *in vivo* and *in vitro*. Our results also showed increased IFN–γ mRNA expression simultaneously in the same embryos that also expressed elevated iNOS mRNA, a macrophage activation marker. This suggested that macrophage activation, subsequent nitric oxide production, and spontaneous embryo loss could be a consequence of local IFN–γ over production.

While LPS serves as an exogenous triggering factor, endogenous TNF–α is known to trigger NO production by primed macrophages. Therefore, we investigated the role of TNF–α, as a second signal, in mediating embryo loss. Our studies showed that the frequency of embryos with significantly increased TNF–α mRNA expression corresponded to the incidence of murine embryo abortion. In addition, the results showed that increased TNF–α mRNA was simultaneously expressed with iNOS mRNA suggesting a potential role for TNF–α in the triggering of decidual macrophages.

In summary, we demonstrated the presence of activated decidual macrophages in murine placentas, and that inducible nitric oxide produced by these macrophages was responsible for embryo death. We further showed that IFN-γ was responsible for the priming of decidual macrophages , and that the expression of TNF-α, a potential secondary signal was associated with decidual macrophage activation, NO production, and subsequent embryo loss.

Abrégé

En utilisant des modèles Murins d'avortement volontaire ou induit, nous avons étudié le rôle que jouent les macrophages dans la perte avancée d'embryons.

Les résultats ont montré que les macrophages produisent de l'oxyde nitrique qui est impliqué dans l'avortement d'embryons. Plus encore, ces macrophages peuvent être activés par la Lippolysaccharride (LPS) pour produire l'oxyde nitrique, indiquant que les cellules mononucleaires ont été pré-activées *in situ*.

En utilisant l'immunocoloration double, nous démontrons que les macrophages sont la source cellulaire de la production induite d'oxyde nitrique provoqué. Nous démontrons aussi que l'avortement des embryons peut-être diminué de manière significative en inhibant la production d'oxyde nitrique *in vivo*.

Les résultats présentés suggèrent que l'oxyde nitrique est une molécule effectrice qui joue un rôle dans la perte avancée d'embryons. Ces résultats suggèrent aussi que l'activation *in situ* des macrophages caducs était un évènement précédant l'avortement volontaire.

Nous savons que l'interferon-γ (IFN-γ) est la cytokine majeure responsable de la pré-activation des macrophages. Nous savons aussi que les LPS peuvent induire des macrophages pré-activés pour produire

de l'oxyde nitrique. Ainsi, le fait que des LPS externes ont induit l'avortement des embryons dans la plupart des lignées de souris enceintes suggère que les macrophages ont été préalablement amorcés *in situ*.

Afin d'examiner le rôle d'IFN- γ comme signal amorceur possible pour l'activation des macrophages caducs, nous avons étudié l'effet de l'épuisement d' IFN- γ sur l'avortement induit par LPS.

Les résultats ont montré que les souris ayant une déficiance en IFN-γ étaient plus resistantes à l'avortement induit par LPS que les souris témoins. Celà suggère qu' IFN- γ était essentiel pour amorcer les macrophages caducs, et que ces macrophages ne pourraient pas être activés lorsqu'exposés au LPS aussi bien *in vivo* qu'*in vitro*.

Nos résultats montraient aussi une expression élevée d'ARNm d'IFN- γ et d'ARNm d'iNOS (marqueur pour l'activation des macrophages) dans les mêmes embryons avortés.

Ces résultats suggèrent que l'activation de macrophage, la production de subséquente d'oxyde nitrique et la perte spontannée d'embryons pourraient être la conséquence d'une surproduction locale d'IFN- γ.

La LPS sert de facteur d'activation externe, tandis que la TNF-α interne est connue pour activer la production d'oxyde nitrique par les macrophages pré-activés.

Ceci dit, nous avons examiné le rôle de la TNF-α, en temps que second signal, dans l'avortement. Nos études ont montré que la fréquence des embryons avec une augmentation élevée de l'expression d'ARNm de TNF-α correspond au taux d'avortement des embryons chez la souris.

De plus, ces résultats ont montré que l'ARNm de TNF-α élevé était simultanément exprimé avec l'ARNm d'iNOS, ce qui suggère un rôle potentiel de la TNF-α dans l'activation des macrophages caducs.

En somme, nous avons démontré la présence de macrophages caducs dans l'interface foeto-maternel. L'oxyde nitrique produit par ces macrophages était responsable pour la mort de l'embryon. Nous avons également montré qu' IFN-γ était responsable pour l'amorçage des macrophages caducs, et que l'expression de TNF-α, un signal secondaire potentiel, était associée à l'activation des macrophages caducs, à la production d'oxyde nitrique et à la perte subséquente de l'embryon.

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First and most of all, I thank Dr. Baines for giving me the opportunity to work in his laboratory. Over the past four years, Mac Baines has guided and directed my research work in a very professional manner. Thank you Mac for your supervision, your friendship, and for the time you have given me.

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Manuscript and Authorship

In accordance with guidelines concerning thesis preparation from the Faculty of Graduate Studies and Research, McGill University, and with the approval of the Department of Microbiology and Immunology, McGill University, I have opted to present the experimental portion of this thesis (Chapters 2, 3, and 4) in the form of manuscripts. The guidelines concerning thesis preparation read as follows:

"Candidates have the option, subject to the approval of their Department, of including, as part of their thesis, copies of the text of a paper(s), submitted for publication, or the clearly- duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.

If this option is chosen, **connecting texts, providing logical bridges between different papers, are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still confirm to all other requirements of the "Guidelines Concerning Thesis Preparation". **The thesis must include:**

A table of contents, an abstract in English and in French, an introduction which clearly states the rationale and objectives of the study, a comprehensive general review of the literature, a final conclusion and summary and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make explicit statement in the thesis of who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such claims at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is the candidate's interest to make perfectly clear the responsibility of different authors of co-authored papers. **Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.**

With regard to the above conditions, I have included as chapters of the thesis one reprint of original paper which have been published (chapter 2), one reprint which has been submitted for publication

(chapter 3), and a second reprint which has been accepted for publication (chapter 4). Chapters 2, 3, and 4 contain their own summary, introduction, materials and methods, results, and discussions and references. A short section which serves as the connecting text to bridge the three chapters is found at the beginning of each chapter. A chapter (chapter 1) which includes introduction and literature review and another (chapter 5) which contains discussion and future directions have been included. References for the literature review follow it directly.

The manuscripts in order of appearance in the thesis, are listed below. The contributions of co-authors are specified under each manuscript. I am responsible for the rest of the work presented in these manuscripts. Composition of the text that appears in the manuscripts was entirely my responsibility, with editing and correction by Dr. M.G. Baines. All the research had been conducted under the supervision of Dr. M.G. Baines.

1. Haddad, E. K., A. J. Duclos, and M. G. Baines. 1995. Early embryo loss is associated with local production of nitric oxide by decidual mononuclear cells. *J. Exp. Med. 182*:1143.

A.J. Duclos provided important discussion, and statistical advice during the preparation of the manuscript. Dr. M. G. Baines, as supervisor provided guidance, advice and was responsible for editing the manuscript.

2. Haddad, E.K., A.J. Duclos, Antecka, E. W.S. Lapp and M. G. Baines Interferon-γ Primes Macrophages for Nitric Oxide production and Fetal Abortion: In Vivo Role of IFN-γ in Early Embryo Loss. Journal of Experimental Medicine (Submitted)

A.J. Duclos did the experiment which involved IFN-γ antibody injections. E. Antecka was responsible for animal matings. W. Lapp provided useful discussion and scientific input. M.G. Baines provided guidance, suggestions, and editing of the manuscript.

3. Haddad, E.K., A.J. Duclos, W.S. Lapp, and M.G. Baines. 1996 Early Embryo Loss is Associated with the Prior Expression of Macrophage Activation Markers in the Decidua. Journal of Immunology (Accepted).

A.J. Duclos provided useful discussion. W.S. Lapp provided the reagent and equipments used in this study as well as scientific input. M.G. Baines provided continuous guidance and suggestions, and editing of the manuscript.

Contribution to Original Knowledge

1. Demonstrated that nitric oxide production by activated decidual macrophages was associated with embryo resorption, and that treating mice with nitric oxide inhibitors such as Aminoguanidine, significantly decreased the incidence of embryo loss. This suggested that nitric oxide is an effector molecule mediating embryo abortion.

2. Demonstrated that mice that lack IFN-γ genes, or mice treated with anti-IFN-γ antibodies were resistant to embryo loss. This indicated that IFN-γ was critical for the occurrence of embryo loss. We further demonstrated that IFN-γ was responsible for the priming of decidual macrophages and nitric oxide production.

3. Demonstrated that TNF-α was associated with early embryo loss. We further showed that TNF-α was associated with macrophage activation and nitric oxide production indicating that TNF–α could act as a second signal to trigger the production of nitric oxide by decidual macrophages.

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List of Abbreviations:

DLN, draining lymph nodes

Dnase, deoxyribonuclease

EDTA, ethylenediaminetetraacetic acid

ELISA, enzyme-linked immunosorbet assay

FCS, fetal calf serum

HIV, human immunodeficieny virus

HLA, human histocompatibility complex

IFN, interferon

IL, interleukin

i.v., intravenous

kD, kilodalton

LGL, large granular lympgocytes

LPS, lipopolysaccharide

mAb, monoclonal antibody

MEM, mimimal essential medium

MHC, major histocompatibility complex

MLR, mixed lymphocyte reaction

NK cell, natural killer cell

PBS, phosphate buffered saline

PCR, polymerase chain reaction

PHA, phytohemagglutinin

Rnase, ribonulease

SCID, severe immunodeficiency

TGF, transforming growth factor

Chapter I

Literature Review:

During mammalian reproduction, allogeneic embryos survive in an immunologically hostile maternal environment by a remarkable but poorly understood mechanism. Even so, few of pregnancies are successful. In fact, in humans as few as 25% of implanted mammalian embryos may survive to term. This report primarily summarizes reviews pertaining to murine reproduction, with occasional reference to human studies as indicated in the text.

Embryo Development

Since most of the discussion is focused on the cellular and molecular events at the fetal-maternal interface, a brief background summary of embryo development helps to put early embryo loss in perspective. After fertilization, the zygote undergoes a series of symmetrical cell divisions that lead to the formation of the morula and later the blastocyst. This process of preimplantation development is followed by implantation where the blastocyst attaches itself to the uterine wall. Preimplantation development is not influenced by the maternal environment. Implantation, on the other hand, promotes thickening of the endometrium and leads to the formation of the decidua, which is the hormonally induced end products of the maternal

myeloid and endometrial cells. The blastocyst capsule contributes the placenta and the inner mass becomes the fetus. Immunohistochemical analysis of frozen sections demonstrated the presence of different immune effector cell populations in the decidua. These include T lymphocytes, natural killer (NK) cells, macrophages, granulocytes, and a cell population restricted to the decidua known as large granular lymphocyte (LGL) in humans, and its equivalent granulated metrial gland (GMG) cells found in mice (Marshall and Jones, 1988; Mincheva-Nilsson et al., 1992; Starkey et al., 1988). In mice and humans, the fetal placental cells or trophoblast line the maternal–blood sinusoids and thus are directly exposed to the maternal immune system (Bischof and Martelli, 1992).

Embryo Loss:

Complications in mammalian pregnancy could occur at any gestational age. There are pregnancies that terminate at the early stages of gestation while others terminate mid-way or even at a later stage of pregnancy. Consequently, the cellular and the molecular factors involved in these complications are different with the different stages of fetal and embryonic development. Spontaneous abortion is one of the most common complications in mammalian pregnancy where the incidence of rate loss can be as high as 60% of implanted embryos

(Roberts et al., 1990). In humans, early embryo loss primarily occurs early in embryonic development, usually 7-14 days following implantation while spontaneous or recurrent abortion occurs at later times (Baines and Gendron, 1993). The reasons for this high incidence of losses has been attributed to several factors, including genetic, endocrine, immune, and many others.

Whereas losses attributed to genetic, anatomic, immune, and endocrine etiologies constitute a large percentage of losses in mammalian pregnancy, about half of the causes remain unidentified. The close interaction between maternal and fetal tissues has suggested that maternal-fetal immune regulation plays critical role in reproductive biology. Data accumulated from human clinical trials and from animal models has strongly suggested a primary role of the immune system in pregnancy outcome.

Immunologic Factors in Spontaneous Abortion

Immunology is the study of the host response that specifically distinguishes "self" versus "non-self". A growing embryo inherits 50% of its genome and antigens from the paternal partner and hence is considered foreign by the maternal immune system and is dealt with as an allograft. The primary role of the maternal immune system in mammalian pregnancy is still debatable. Some have suggested that an

immune response is essential for a successful pregnancy (Athanassakis et al., 1987), while others showed that an immune response generated to fetal antigens may induce negative reproductive outcome (Redman, 1993; Scott et al., 1987; Khong, 1989). Spontaneous abortion is the ultimate terminal complication of mammalian pregnancy. Many hypotheses have been postulated to explain the role of immunological mechanisms in pregnancy loss.

Major Histocompatibility Complex Antigens

The major histocompatibility complex (MHC) antigens expressed by tissue cells were originally described as being the target structures responsible for the rejection of tissue or organ grafts. The role of the MHC proteins in pregnancy has long puzzled immunologists and its roles in pregnancy remain controversial. During infections, autologous MHC antigens expressed on antigen presenting cells are responsible for presenting foreign peptides to lymphocytes. In the context of allograft rejection, host T-cells bind to graft MHC directly initiating a powerful cell mediated immune response leading to the rejection of the graft. By the same token, MHC alloantigens present on fetal cells, may induce a maternal immune response against the growing embryo causing its rejection. Conversely, it is clear that in most cases maternal

alloimmunization against paternal MHC has no deleterious effect on pregnancy outcome and may even be beneficial (Beer et al., 1981).

MHC expression in the placenta has been studied both in humans as well as in mice. Placental tissue sections taken from allogeneic (CBA females mated with Balb/c males) matings were stained with antibodies to paternal MHC class I and class II antigens. The results showed that a significant proportion of trophoblast cells expressed class I antigens of both parental haplotypes. These results indicated that MHC class I antigens of paternal origin were expressed by trophoblast cells at the maternal interface. However, the presence of MHC class II antigens has not been detected (Lala et al., 1983c; Chatterjee-Hasrouni and Lala, 1982). Further studies were conducted to determine whether MHC class I antigens were accessible to the maternal immune system, and consequently were able to stimulate a maternal allo-immune response. Unexpectedly, the results showed that the expression of MHC class I antigens in the placenta was on those cells that were directly exposed to the maternal blood circulation (Lala and Kearns, 1985). The results further showed that placental cells positive for MHC class I expression were able to induce an immune response *in vitro* (Lala et al., 1983b). Hence in the mouse, the fetal tissues were exposed to the maternal immune response, yet the fetus managed to survive. One speculative role of these MHC class I antigens is to adsorb maternal antibodies

directed against paternal MHC antigens and hence, enhancing pregnancy outcome (Lala et al., 1983b). Alternatively, MHC expression may be essential for preventing natural killer cell mediated lysis of trophoblasts (Kane and Clark, 1986).

In humans, studies have shown that there was an absence of MHC class I and class II antigens expression on placental cells in direct contact with maternal blood (Hunt et al., 1988; Sunderland et al., 1981; Faulk and Temple, 1976). However, a monomorphic MHC class I antigen has been shown to be expressed in the placenta (Kovats et al., 1990; Ellis et al., 1990). This antigen known as HLA-G has been identified by monoclonal antibodies directed against the conserved region of the conventional HLA-A,B, and C. HLA-G has been cloned and its expression has been shown to be restricted to the placental tissues suggesting its involvement in reproduction (Shukla et al., 1990). The role of HLA-G in promoting embryo survival is not well understood. However, transfection experiments have shown that cells transfected with HLA-G were significantly less sensitive to NK cell killing than mock transfected controls lacking MHC-I (Kovatts et al., 1991). This indicated that HLA-G might inhibit NK cell activation and hence protect against NK induced embryo death. It has been further shown that HLA-G binds to CD8 receptors found on T-cells suggesting that HLA-G might have a competitive role in the recognition of foreign intracellular

antigens(Sanders et al., 1991). In mice, non-polymorphic MHC class I (Qa and T1) antigens have also been identified on fetal placental cells. These antigens have been shown to bind to the specific receptors of γ/δ T-cells (Vidovic et al., 1989).

Further insight into the role of MHC in mammalian pregnancy has been revealed by investigating cases of couples experiencing recurrent spontaneous abortion of unexplained origin. Recurrent spontaneous abortion has been associated with the sharing of HLA (MHC-II) antigens between husband and wife (Ober et al., 1983, 1984). In cases of recurrent abortion, immunizing the wife with paternal leukocytes often helped to enhance embryo survival (McIntyre and Faulk, 1983). However, detailed statistical evaluation of these clinical studies revealed that this treatment had limited success since placebo or psychological treatments such as enhanced patient support alone, were also found to have high success rates (Anonymous, 1994). Furthermore, not all controlled trials have confirmed the previous beneficial results (Hill, 1991). It has been shown that couples who share a limited diversity of MHC haplotypes, such as the Hutterites, have longer time to pregnancy compared to non-matched couples of the same community (Ober et al., 1983, Nonaka et al., 1994). However, when studies were performed with the proper population-based control, the results demonstrated that HLA heterogeneity is not critical for a normal pregnancy. Rather, such factors

have a slight but significant negative effect on fertility, but apparently not on embryo survival (Stray-Pedersen and Stray-Pedersen, 1984; Ober et al., 1985)

Likewise, studies in inbred mice have shown that sharing MHC haplotypes is not detrimental to pregnancy. For instance, inbred mice which are homozygous at all loci on both haploid sets of chromosomes do not normally experience a significant incidence of embryo wastage. Further evidence derived from transgenic mice have revealed that mice deficient in either MHC class I or II breed normally and their offspring survived without any obvious morphogenetic defects (Grusby et al., 1991; Zijlstra et al., 1990). These experiments showed not only that diversity of expression of MHC antigens is not a requirement for fetal survival, but also that MHC expression is not even needed for successful pregnancy. Conversely, normal or increased MHC expression does not induce pregnancy loss.

Suppressor Cells

The absence of suppressor cells and suppressor factors is another immune mechanism suggested as a possible cause for spontaneous abortion. If normal pregnancy could be considered as an example of prolonged acceptance of an allograft, spontaneous abortion could represent maternal rejection. The hypothesis that immune suppression

exists during pregnancy derives primarily from a need to explain the failure of the maternal immune system to reject the fetal allograft. For this reason, experiments have been conducted to identify the nature of the suppressor cells as well as the suppressor factors involved. Evidence for the existence of suppressor cells have been reported by Clark and his colleagues in the late 70s and early 80s (Clark et al., 1986; Clark and McDermott, 1978, Lachapelle et al., 1996). They studied the immunoregulatory functions of lymph node cells draining the uteri (DLN) of allogeneic pregnancies. They showed that DLN taken from C3H/HeJ ($H2^k$) females mated with DBA/2J($H2^d$) expressed suppressive activities when injected into newborn mice where a fetal graft versus host reaction was the expected outcome (Clark and McDermott, 1978). First, DLN from allogeneic pregnancy had reduced ability to kill C3H X DBA/2 F1 new born mice when compared to DLN from virgin mice (Clark and McDermott, 1978). This reduction in mortality was correlated with a diminished CTL cytotoxic activity in the spleens taken from the injected new born mice. Moreover, both *in vivo* and *in vitro* experiments showed that DLN taken from allogeneic pregnancy possessed an impaired ability to generate CTL against paternal antigens. These same suppressive cells were later shown to inhibit NK cell activation and con A induced stimulation of T-cells *in vitro* (Clark et al., 1986). Further experiments were conducted to identify the nature of these suppressor

cells. These studies demonstrated that the DLN suppressive cells were non-thymus derived and showed antigen non-specific activities. Further evidence for the existence of suppressor cells was demonstrated in mice and in humans. It was shown that in mice, suppressor activities were absent in DLN from high loss pregnancies such as in CBA/J females mated with DBA/2 males, and from amniotic fluids of aborted pregnancies (Clark et al., 1986). It was later shown that the suppressor cells produced TGF-β like immunosuppressive factors which were able to inhibit the activation of T-cells and NK cells (Clark et al., 1994).

Alpha-fetoprotein (AFP) is another suppressor factor that has also been shown to be associated with embryo survival by inducing a state of immune suppression at the fetomaternal interface (Murgita, 1976). AFP has been shown to inhibit cell mediated immune responses (Cohen et al., 1986; Hooper et al., 1982; Tomasi, Jr. et al., 1975), therefore, the presence of AFP only during fetal life and not during adult life suggested a role for AFP in suppressing maternal immune responses and maintaining normal pregnancy.

Further evidence which support the contention of a local immunosuppressive state in the placenta has been obtained from a murine model of infection during pregnancy with *Listeria monocytogenes*. Studies have demonstrated that normal non-pregnant Listeria resistant mice were easily able to clear systemic infections by

this bacteria. On the other hand, clearance of placental infection was impaired in pregnant mice. This might be due the inability of macrophages and lymphocytes to reach the foci of infection or it might be due to the activation of a Th2 cytokine response that augmented antibody mediated immunity and reduced local cell mediated immunity (Ahlfors et al., 1977; Redline and Lu, 1987, Redline and Lu, 1988).

However, since they were initially reported in the 70s, suppressor cells have usually been described as activities of cells with a number of different phenotypes and a clear characterization of these cells is still lacking.

Studies in humans have shown that a deficiency of suppressive activity resulted in many early pregnancy failures following *in vitro* fertilization and embryo transfer (Nobel, 1984) . More insights into the mechanisms of immunosuppression in mammalian reproduction can be elucidated by studying the changes of the maternal immune function that occur during pregnancy. Many observations have suggested that the maternal systemic and local immune responses decline during pregnancy. For instance, during pregnancy the mother is more susceptible to some infectious diseases. It has been observed that during pregnancy, latent infectious agents such as cytomegalovirus (CMV), and Listeria may become active (McLauchlin, 1990; Weinberg, 1984). In the same context, it has been shown that during pregnancy

some cell mediated autoimmune diseases improve. However, these observations have been contradicted by data which demonstrated that many other infectious diseases were not reactivated or more severe in pregnant females (Evron et al., 1984). It was further shown that some antibody mediated autoimmune diseases such as systemic lupus erythematosus (SLE) may worsen during pregnancy while still others remained unaffected (El-Roeiy and Shoenfeld, 1985; Lockshin, 1985). Studies concerning alterations in systemic maternal cellular and humoral immunity during pregnancy have not been very supportive of the immunosuppressive theory. It has been shown that B-cell and T-cell numbers as well as functions were not significantly changed (Sridama et al., 1982; Moore et al., 1983). Some decrease in NK cell activities has been reported (Toder et al., 1984). Moreover, compelling evidence of normal maternal response to vaccines and ability to mount a delayed-type hypersensitivity (DTH) response argued against a state of systemic immunosuppression during pregnancy (Murray et al., 1979, Lichtenstein, 1942).

Overall, over the past two decades the issue of immunosuppression in pregnancy has been controversial. Further, the phenotype of suppressor cells has never been conclusively identified, and a state of total immunosuppression has never been demonstrated both systemically or locally. It could be that during pregnancy there is a

profound increase in local steroid hormones or suppressive cytokines which may induce a local state of suppression which could explain the survival of the conceptus during pregnancy.

Antibody mediated spontaneous abortion

Immune responses are normally stimulated when an individual is exposed to foreign antigens. These immune responses are classified as being cellular or humoral. During pregnancy, the allogeneic conceptus stimulates the maternal humoral immune responses. It has been postulated that the rejection of the conceptus by the mother is due to the enhanced generation of antibodies against paternal antigens. Different types of antibodies have been described in the literature, such as anti-phospholipid antibodies, anti-nuclear antibodies, and possibly anti-sperm antibodies. The survival of the embryo on the other hand, has been assumed to be due to the blocking of the maternal anti-paternal cell mediated immune responses by the generation of blocking antibodies. Blocking antibodies may mask paternal antigens without initiating cytopathology.

As the name indicates, anti-sperm antibodies react to antigens found on the surface membrane of sperm cells. The first indication of the involvement of antisperm antibodies in embryo loss was demonstrated in the early 70s (Menge, 1970; Haas, Jr. et al., 1986). The

experiments showed that antisperm antibodies generated in female mice after immunization with semen exerted embryocytotoxic effects. It is believed that antibodies to sperm antigen cross react with antigens found on the growing embryo causing its death. These results were further substantiated by studies derived from human pregnancies. It was shown that anti-sperm antibodies had been found in 10%-40% of women experiencing spontaneous abortion of unknown origin. However, these results were disputed by recent data that showed that less that 0.5% of women experiencing spontaneous embryo loss were found to have antisperm antibodies (Naz et al., 1990; Yan et al., 1990). Recent data further showed that anti-sperm antibodies have also been found in women with successful pregnancies (Jager et al., 1984; Clarke and Baker, 1993). It was further shown that anti-sperm antibodies had no effect on embryo survival since they did not induce embryo abortion in pregnancy resulting from *in vitro* fertilization technology (Yan et al., 1990). These results indicated that antisperm antibodies were unlikely to be involved in pregnancy failure.

Antiphospholipids antibodies are antibodies that are directed against negatively charged phospholipids (Harris et al., 1988). They comprise a group of heterogeneous antibodies such as Lupus anticoagulant and anticardiolipin antibodies that are found in antiserum of patients with the autoimmune disease, systemic lupus

erythematosus (SLE) (Sthoeger et al., 1993). These antibodies have been shown to be associated with thrombosis and thrombocytopenia (Jungers et al., 1984; Oksenhendler et al., 1984). Despite their close correlation with SLE patients, their roles in the disease are still not clear as they may be a result of SLE induced cytopathology and not the primary cause of cell damage. However, there is strong evidence that patients with SLE experience difficulties in both conception and maintenance of pregnancy (Harris et al., 1985).

The most specific clinical feature of the presence of anti-phospholipid antibodies is pregnancy loss. For many years, it has been shown that antiphospholipid antibodies were consistently present in the serum of women experiencing repeated embryo loss of unexplained origins (Harris et al., 1985; Sammaritano et al., 1990; Deleze et al., 1989; Kwak et al., 1994). This circumstantial evidence prompted further experiments to identify the role, if any, of these antibodies in repeated pregnancy failure. Studies in humans have shown contradictory results. Some reports indicated that women with repeated pregnancy failure showed significantly higher incidence of antiphospholipid antibodies than normal controls (Lubbe and Liggins, 1985). Many other studies confirmed these results, and further showed that down regulation of maternal antiphospholipid antibodies during early pregnancy could prevent embryo loss (Kwak et al., 1992). Other studies have shown that

mice passively immunized with monoclonal or polyclonal antibodies against cardiolipin showed an increased incidence of embryo loss (Blank et al., 1991). On the other hand, these results were not substantiated by other investigators (Makino, et al., 1992). Further, anti-phospholipid antibodies have been found in women with normal pregnancies as well as in non-pregnant women (Kwak et al., 1992). Taken together, these results showed that anti-phospholipid antibodies are weak indicators of repeated abortion (Coulam, 1992).

Another antibody mediated mechanism for repeated abortion has been shown to involve blocking factor deficiency. Blocking factors were shown to block the maternal anti-paternal immune responses in mixed lymphocyte reactions (MLR). It was shown that maternal serum factors were able to inhibit a MLR of maternal responder against paternal stimulator cells (Beer et al., 1981; McIntyre and Faulk, 1983). These blocking factors have later been shown to be absent in the antiserum of women with a history of repeated abortion (Rocklin et al., 1976). However, there is no direct evidence that these blocking factors are found in all successful pregnancies (Rocklin et al., 1982). In fact, studies have shown that blocking serum factors were present in some cases of repeated abortion, and absent in some normal pregnancies. These immunoglobulin factors had never been characterized as to specificity, and hence their involvement in repeated abortion is questionable.

TH1/TH2 Cell mediated mechanism in pregnancy

Cell mediated immune responses constitute the second arm of the specific immune systems. T-cells are either CD4+ or CD8+ cells and CD4+ T-cells recognize antigens in the context of MHC class II antigens, while CD8+ cells recognize antigens in the context of MHC class I antigens (Yewdell and Bennink, 1992; Madden, 1995). Antigen activated CD4+ T-cells produce soluble factors or cytokines that activate B-cells to produce antibodies or they also produce cytokines that activate a variety of effector cells such as cytotoxic CD8+ T-cells, macrophages and NK cells. *In vitro* and later *in vivo* studies have shown that CD4+ T-cells could further sub-classified to TH0, TH1, and TH2 cells depending on the profile of cytokines they produced (Mosmann et al., 1986; Street and Mosmann, 1991; Janeway, Jr. et al., 1988; Mosmann and Coffman, 1989). In general TH1 cells produced primarily interferon-γ (IFN-γ), tumor necrosis factor-β (TNF-β), and IL-2, while TH2 cells produced IL-3, IL-4, IL-10 and other cytokines (Romagnani, 1994; Seder and Paul, 1994). TH0 are intermediate cells or precursors of TH1 and TH2 and can produce limited quantities of both cytokine profiles. Therefore, TH1 cytokines such as IL-2 and IFN-γ induce cell mediated cytotoxic activities by activating CD8+ T-cells, NK cells and macrophages (Cox et al., 1990; Narumi et al., 1990), while the TH2 cytokines, IL-4 and IL-10, inhibit TH1 cytokine production and hence suppress the generation of

cell mediated cytotoxcicity (McBride et al., 1990; Cox et al., 1991). It is not yet known what induces TH0 cells to become either TH1 or TH2. Studies have shown that a variety of factors may be involved such as antigen presentation (Villacres-Eriksson, 1995), presence of co-stimulatory factors (Kuchroo et al., 1995), and even the presence of traces of IFN-γ and IL-4 before the initiation of the immune response (Ferrick et al., 1995). This switch point is a very critical stage in combating diseases since in some cases, TH1 cytokines are associated with resistance to a disease such as Leishmenia, while TH2 cytokines may increase susceptibility to disease, and *vice versa* in the case of other diseases (Fitzgerald, 1992; Chatelain et al., 1992).

The overproduction of TH1 cytokines or a deficiency in TH2 cytokines during pregnancy has been proposed as one mechanism which favor spontaneous abortion. The TH2/TH1 nature of the cytokines produced determines the success or failure of a conceptus as TH2 cytokines enhance fetal survival (Wegmann et al., 1993; Hill et al., 1995). Events which increase local TH1 cytokine production at the fetomaternal interface could augment cell mediated immunity generated against fetal antigens and threatened fetal survival.

The local presence of TH1 and TH2 cytokines have been studied in many laboratories and the effect of these cytokines on local cellular

responses in the deciduum and consequent early embryo loss have been previously described (Krishnan et al., 1996b; Krishnan et al., 1996a).

Immune response Initiators and early embryo loss

T-lymphocytes

Mature T-cells which express a specific T cell receptor (TCR) are either CD4+/CD8- or CD8+/CD4-. These cells are able to initiate a specific immune response in response to foreign antigens in the presence of MHC class I or class II. It has been postulated that local specific immune responses could be initiated against fetal antigens in the decidua leading to T-cell activation and production of cytokines. The nature of these cytokines determines what effect they have on pregnancy outcome. As previously stated, TH1 cytokines such as IFN-γ and TNF-β have deleterious effect on the embryo while TH2 cytokines such as IL-4, IL-10 and IL-3 are useful to pregnancy .

Many studies have sought to quantify the presence of T-cells at the fetomaternal interface and showed that cells bearing these T-cell markers were sparsely present in both human and murine decidua. In humans, the data showed the presence of CD8+ cells at all stages of pregnancy (Mincheva-Nilsson et al., 1994), but there was an occasional presence of only CD4+ T-cells in the deciduum (Bernard et al., 1978).

However, the results from murine pregnancies regarding the expression of CD4 and CD8 markers remaincontroversial. While some have reported the presence of CD8+ cells detected early in pregnancy, other studies have indicated the absence of these cells (Lala et al., 1983a). Further analysis of CD8+ cells has shown that they also expressed CD2 and Asialo-GM1, but not CD3 antigens, suggesting that these cells were NK-like cells and not classical cytotoxic T-lymphocytes (CTL) normally observed in lymphoid tissues (Head et al., 1994). The expression of CD4+ cells has been shown to be very rare in early pregnancy but may become more common during early embryo loss (Gendron and Baines, 1989)

T-cells with α/β T-Cell Receptors

The role of α/β T-cells cells in mediating repeated abortion is not clear. Data for many laboratories support the contention that α/β T-cells do not play a primary role in mediating early embryo failure. First of all, the presence of these cells has been disputed. Most of the studies done on CD4 and CD8 expression were done prior to the description of CD4+ or CD8+ T-cells expressing γ/δ TCR. It may also be significant that macrophages bear the CD4 receptor. More significant, is the absence of these cell populations in early pregnancy, the time when events leading to the recognition of the fetal antigens should be taking place (Lala et

al., 1983a; Bernard et al., 1978). The most conclusive data regarding the insignificance of the role of α/β T-cells in mediating abortion has come from studying mice that are naturally deficient in mature α/β T-cells (Dosch et al., 1978; Bosma et al., 1983). These mice known as nude and SCID mice, have been shown to be capable of rejecting grafted xenogeneic embryos indicating that different and probably non-specific cellular components of the host were capable of chronic graft rejection and consequently embryo loss.

In mice, it is now very well documented that fetal villous syncytial trophoblast cells facing the maternal immune cells do not express polymorphic MHC class I and class II antigens (Croy et al., 1985b; Crepeau and Croy, 1988). Therefore, it seems unnecessary to assign a functional role of α/β T-cells in preventing pregnancy failure. On the other hand, the expression of non-polymorphic MHC antigens on cells of fetal origin strongly suggest that cells with non-conventional antigen receptors such as T-cells with γ/δ T-cell receptors and NK cells may be involved in recognizing fetal antigens.

These results showed that α/β T-cells are unlikely to mediate embryo loss. The absence of MHC antigens would argue against specific recognition events involving these cells. Further, the SCID experiments indicated that T-lymphocytes were not needed to mediate effector functions leading to embryo rejection.

T lymphocytes with γ/δ Receptors

For a long time, it has been known that the vast majority of T-cells in lymphoid tissues bear the α/β TCR in association with the invariant component of CD3 complex. It is now known that a minority of T-cells express alternative TCR composed of γ and δ chains, that are also associated with the invariant CD3 complex (Fowlkes and Pardoll, 1989; Raulet, 1989; Allison and Raulet, 1990). Since their discovery, little is known regarding their repertoire and effector functions. T-cells bearing the TCR γ/δ chains have been shown to be the first T-cells to appear in ontogeny and that α/β chain rearrangements result in deletion of the γ/δ genes (Bluestone et al., 1987; Pardoll et al., 1987; Havran and Allison, 1988).

The majority of γ/δ T-cells are found in epithelial cells. Still, a minority of γ/δ T-cells are found in the blood (Allison and Havran, 1991). Unlike the circulating γ/δ and α/β T-cells, epithelial γ/δ T-cells exhibit highly restricted variable domains and hence lack the TCR diversity common to T-cells expressing α/β TCR (Koning et al., 1988b; Koning et al., 1988a; Stingl et al., 1987). Epithelial γ/δ T-cells have been shown to be selectively expressed in the skin, intestinal epithelium, lung, and the uterus (Tschachler et al., 1983; Itohara et al., 1990). What functions these cells serve at these sites are unknown. These cells have been shown to be associated with different viral, bacterial, and autoimmune

diseases, suggesting that these cells might be important in regulating immune responses to these diseases (Haas et al., 1993; Born et al., 1990).

In the uterus, γ/δ T-cells are referred to as reproductive-γ/δ T-cells (r-γ/δ). In mice, these cells have been shown to be expressed in pregnant as well as in non-pregnant uteri (Heyborne et al., 1992; Meeusen et al., 1993). Data concerning the presence of γ/δ T-cells in human decidua is still controversial. While some studies (Mincheva-Nilsson et al.,1992) have reported the presence of these cells in human uteri, other studies were not able to corborate these observations (Vassiliadou and Bulmer, 1996). Studies have shown that r-γ/δ TCR have no junctional diversity and hence they have restricted polymorphism to recognize foreign antigens. These cells have been under intense investigation by reproductive immunologists in an attempt to elucidate their function in mammalian pregnancy. Immunohistochemical and flow cytometric studies done on decidual leukocytes from normal human pregnancies have shown that TCR γ/δ expressing cells comprised about 60% of the uterine T-cells. Some of them were CD4+ and others are CD8+, but the majority are CD4- and CD8-. Interestingly, 30% to 58% of these cells express the CD45RO antigen, a marker for primed or activated T-cells (Mincheva-Nilsson et al., 1992). It has been further confirmed that r-γ/δ T-cells expressed the high affinity IL-2 receptor, another T-cell activation

marker (Heyborne et al., 1992). More importantly, these studies have shown that the activation of r-γ/δ T-cells was only observed in pregnant but not in non-pregnant uteri. This suggested that r-γ/δ T-cells may have been involved in regulating a variety of physiologic events in reproduction (Nandi and Allison, 1991). However, it has also been observed that r-γ/δ T-cells showed poor responsiveness to stimuli such as alloantigens and anti-CD3 antibodies, indicating a limited responsiveness or state of immunosuppression (Mincheva-Nilsson et al., 1992). Although r-γ/δ T-cells express activation markers, they have been shown to be unresponsive to alloantigens due to unknown mechanisms which prevent an immunological rejection response during normal pregnancy (Mincheva-Nilsson et al., 1992). It could be that in early embryo loss, r-γ/δ T-cells are either absent or are not suppressed and hence can mediate effector functions against the allogeneic fetal cells.

In order for r-γ/δ T-cells to initiate and mediate immune responses against allogeneic fetal cells, they should be able to recognize paternal or trophoblastic antigens as well as be able to respond to these stimuli by producing effector molecules. The ligands for γ/δ TCR are not well defined, however, it is very well established that r-γ/δ T-cells possess non-MHC restricted recognition, suggesting antigen presentation receptors different from MHC are needed for r-γ/δ T-cell antigen

recognition (Matis and Bluestone, 1991; Montesoro et al., 1991; Iwashima et al., 1991).

Earlier studies have shown that peripheral γ/δ T-cells can be stimulated by non-protein antigens derived from bacterial cell walls (Tsuyuguchi et al., 1991; Fleischer, 1991; Griffin et al., 1991). Interestingly, it has been shown recently that r-γ/δ T-cells recognize trophoblast associated heat shock proteins (Doherty et al., 1991; O'Brien and Born, 1991; Kaufmann et al., 1991). This was shown in mice deficient in MHC antigens confirming previous results regarding non-restriction of r-γ/δ T-cells to MHC antigens. Studies have also shown that certain γ/δ T-cell receptors bind to non-polymorphic MHC class I antigens expressed mostly in human and murine implantation sites further supporting the importance of these cells in mammalian reproduction (Vidovic et al., 1989). Recently, our laboratory investigated the role of heat shock proteins in early embryo loss. The results showed that there was an increased and selective expression of some heat shock proteins in resorbing but not in healthy embryos (Duclos et al., 1996). Collectively, these results indicated that r-γ/δ T-cells can recognize trophoblastic antigens and can initiate an immune response. The nature of this immune response is unknown at this time but is receiving increased attention.

It has recently been shown that stimulated murine r-γ/δ T-cells produced TGF–β, a molecule that was shown to inhibit T-cell dependent immune responses, and hence the role of r-γ/δ T-cells may be to suppress the maternal anti-fetal immune response and prevent rejection of the fetus. On the other hand, stimulated r-γ/δ T-cells could also produce TH1 cytokines such as IFN–γ that lead to macrophage activation and possibly embryo death (Mizoguchi et al., 1996; Ladel et al., 1996). The TH1/TH2 cytokine production by γ/δ T-cells has been shown to play an important role in regulating immune responses against many bacterial and viral infections, but has not yet been demonstrated to affect fetal survival (Nilssen et al., 1996; Hara et al., 1996; Hess et al., 1996).

These studies suggested that r-γ/δ T-cells may play a critical role in normal pregnancy by either maintaining normal physiological function at the fetomaternal interface or by inducing embryo loss. The results further showed r-γ/δ T-cells could initiate these responses through their ability to produce TH1 as well as TH2 cytokines. TH1 cytokines could then induce a deleterious cell mediated immune responses leading to embryo death, while TH2 cytokines could suppress cell mediated immune responses to the embryo. It is not known what activates the r-γ/δ T-cells to produce any of these cytokines. Studies are underway to explore the different possibilities.

Animal model of early embryo loss

In humans, early embryo loss may be defined as losses that occur before the third week of gestation. Since these losses often occur before the end of the menstrual cycle, early cellular and molecular events that lead to embryo wastage could not be investigated. Furthermore, it is extremely difficult to study the effect of treatments for repeated abortion in humans as the identification of patients who can be predicted to abort is virtually impossible. For these reasons, animal models have been shown to be invaluable in studying acute or repeated abortion.

Many murine models of induced or spontaneous abortion had been described in the literature. Murine pregnancy shows cellular and molecular characteristics similar to those seen in human pregnancy. The murine gestation period is relatively short and hence allows feasible experimental designs. Unlike human pregnancy, in which the single embryo survives or is aborted, in mice which gestate litters of 7-12 pups, partial abortion or resorption of 2 to 3 embryos per uterus often occurs. In addition, in murine reproduction, the early conceptus is not expelled as in humans, but is retained in the uterus and is resorbed. Later fetal deaths are stillborn with their living littermates.

Mouse Models of Spontaneous Abortion

Many murine models of spontaneous abortion have been described in the literature. For instance, CBA/J females mated with DBA/2 males (Clark et al., 1980) and B10 females mated with B10.A males (Melnick et al., 1981), have both been shown to have high incidence of embryo loss in the ranges from 20% to 30% by day 12 of gestation. The use of inbred mice to study pregnancy failure eliminates many anatomic, genetic and hormonal differences observed in outbred matings, thus allowing investigators to focus on the immunological etiologies of abortion. The study of the CBA/J X DBA/2 model has provided major contributions to our understanding of the mechanisms of early embryo loss. One important finding involved the demonstration that vaccination of the CBA/J females with a paternal lymphocyte or even a third party splenocytes before mating significantly diminished early embryo loss (Chaouat et al., 1983; Kiger et al., 1985). As have been discussed in detail below, elements of the innate resistance system such as NK cells and macrophages were shown to be the primary factors in mediating embryo abortion.

Mouse Models of Induced Abortion

Despite the critical findings demonstrated by studying the naturally resorbing CBA/J X DBA/2 mouse model, the universal role of the cellular and molecular effectors, such as NK cells and macrophages,

in early embryo loss has been greatly facilitated by studying abortion in normal and low loss mouse mating combinations.

The LPS induced early embryo loss model has been invaluable in studying murine abortion in a variety of mouse strains. Lipopolysaccharide (LPS) is found on the outer surface of gram negative bacteria cell membrane. When LPS is injected into a variety of mouse strains during pregnancy, there is 5 to 10 fold increase in the incidence of embryo loss (Gendron et al., 1990, Duclos et al., 1996). Although LPS has been known to be a powerful B-cell and macrophage activator, the mechanisms of LPS induced embryo abortion is not fully understood. It has been postulated that LPS induced embryo abortion by activating macrophages to produce effector molecules such as TNF-α and nitric oxide. Further insight into the mechanisms of LPS induced early embryo loss are discussed in chapters 2, 3, and 4 which indicat that LPS could trigger IFN-γ primed decidual macrophages to produce nitric oxide and subsequent embryo death.

Treating pregnant mice with Poly I:C, a double stranded RNA and an established NK cell activator, has also been shown to induce embryo abortion in most low loss matings 2 to 3 fold (de Fougerolles and Baines, 1987; Kinsky et al., 1990). The Poly I:C model has been used to investigate the role of NK cells and elements of innate resistance in murine abortion. Chapter 3 clearly indicates that Poly I:C induced

embryo abortion is dependent on the presence of IFN-γ. Therefore, Poly I:C injected into mice could induce the production of IFN-γ by decidual NK cells which in turn would prime decidual macrophages for nitric oxide and TNF-α production and embryo loss.

Cellular and Molecular Effector Factors in Natural and Induced Murine Matings

Cellular Effectors

Natural Killer (NK) Cells and NK-Like Cells

Natural Killer cells are CD3- CD16+ CD56+ lymphocytes that are different from T-cells and B-cells, exert cell-mediated cytotoxcicity, and produce cytokines when activated (Trinchieri, 1989; Ortaldo, 1991; Murphy, 1985). NK cells are part of the non-specific or innate resistance system protecting against disease and cancer. They are the first line of defense against invading pathogens and tumor cells until a specific immune response is initiated. The mechanisms by which NK cells recognize and kill their targets are currently being investigated in many laboratories. It is very well documented that NK cells kill their target cells in non-MHC restricted manner. It had been postulated that NK

receptors, other than MHC molecules, mediated NK cell recognition to foreign cellular antigens (Roth et al., 1994; Ferrini et al., 1994).

It has been shown that NK cells kill target cells by two different mechanisms. The granule exocytosis mechanism, where the killing is mediated by the formation of holes in the cell membranes of the target cells through the production of perforin and granzymes (van den Broek et al., 1995). Sufficient perforin to introduce many pores can kill by osmotic lysis within 2-4 hours (Berthou et al., 1995). The entry of the granzymes into the target cell through the perforin pores initiates apoptotic death within 24 hours (Bochan et al., 1995). The other mechanism is a ligand-receptor mechanism where killing of target cells is mediated by cytotoxic molecules binding to their receptors, such as TNF–α produced by NK cells binds to TNF–α receptor on target cells which likewise initiates apoptotic death (Naume et al., 1991; Ikuta et al., 1991).

The role of NK cells in mammalian pregnancy has been studied extensively over the past two decades. However, conclusive results concerning the primary roles of NK cells at the fetomaternal interface are still lacking. Is the early presence of decidual NK cells essential for successful pregnancy, or is the appearance of NK cells detrimental to the fetal survival?

The presence of NK cells at the decidua is measured either by identifying NK specific surface markers or by determining NK-mediated cytolytic activities against known NK targets such as the T-cell lymphoma line , YAC-1 cells. Using these criteria, it has been shown that during very early pregnancy the murine decidua contained significant numbers of NK cells and high levels of NK cytolytic activity when tested against Yac-1 target cells. This activity decreased at mid gestation and became undetectable at the latter half of pregnancy (Gambel et al., 1985; Croy et al., 1985a).

It has been postulated that the presence of NK-like cells at implantation sites is necessary for normal pregnancy. It is believed that NK cells are related to a population of uterine cells known as granulated metrial gland cells (GMG) that are present in the decidualised endometrium of all mammalian species studied thus far (Bulmer et al., 1987; Croy and Kiso, 1993). GMG cells expressed a characteristic marker of mouse NK cells known as asialo-GM1, and lacked markers for T-cells and B-cells including TCR and CD3 receptors (Stewart, 1994; Peel and Adam, 1991). It was further shown, by using lethally irradiated bone marrow reconstituted mice, that GMG cells originated from lymphoid precursors (Peel and Stewart, 1984). Further evidence has shown that GMG cells are not a type of NK cells. First, it has been shown that GMG cells lack some membrane receptors such as the LGL-

1 and NK-1.1/1.2 which were specific for NK cells (Parr et al., 1990). Second, GMG cells were proved to be cytotoxic in Beige mice, which had a genetic defect in NK activity. Third, GMG cells were not able to kill the traditional NK targets (Stewart, 1994, Croy, 1994). These results indicated that GMG cells were different and not a type of NK cells.

Studies have shown that, in the presence of IL-2, mouse GMG and NK cells were cytotoxic to placental trophoblast cells (Linnemeyer, and Pollack, 1991), and that GMG and NK cells contained cytotoxic granules such as perforin, suggesting that the GMG cells could prevent normal placental development (Lin et al., 1991). The functions of GMG cells and NK cells have been shown to be associated with the killing of abnormal placental tissues, and controlling trophoblast invasions during normal pregnancy. It has been further postulated that NK cells and GMG cells protected the embryo from different viral and bacterial infections (Croy et al., 1991). The role of NK cells and GMG cells in normal pregnancy has been disputed by many *in vivo* experiments. It has been shown that Beige mice, which showed genetic defect in NK cells activity, had normal pregnancies (MacDougall et al., 1990). Recently, perforin knockout mice have been generated, and the results showed that perforin deficient mice reproduce normally (Kagi et al., 1994). However TgE26 mice, a newly generated transgenic mice that completely lack NK cells and GMG cells, showed profound reproductive complications. Therefore, GMG cells may

be essential for normal pregnancy and embryo survival (Guimond et al., 1996).

It has been assumed that NK cells were associated with embryo loss. Studies performed on the high loss murine mating model (CBA/J X DBA/2) have shown that the number of asialo-GM1 positive cells was significantly higher in resorbing compared to non-resorbing embryos (Gendron et al., 1990). Further *in vivo* experiments have shown that depleting NK cells with anti-asialo-GM1 antiserum enhanced embryo survival (de Fougerolles and Baines, 1987). Significantly, treatments of pregnant mice with reagents that induce NK cells activity have been shown to enhance early embryo losses. For instance, the incidence of embryo loss in mice injected with Poly I:C, double stranded RNA known to induce interferon-mediated activation of NK cell activity, was significantly increased 2-3 fold (Kinsky et al., 1990; Gendron and Baines, 1989). Likewise, it has been shown that IL-2 treated pregnant mice also express a high incidence embryo loss (Toder et al., 1991, Baines et al., 1997).

The mechanisms by which NK cells exert their cytolytic activities against the growing embryo are not fully understood. It could be that NK cells induce embryo death through the production of perforin-mediated lysis of trophoblasts, however studies using high loss mating models indicate that perforin is equally expressed in both resorbing and non-

resorbing embryos. Alternatively, NK cells may kill embryonic tissues through TNF–α production leading to TNF-α receptor mediated killing of trophoblasts (Lindemann, 1991). Nevertheless, it has been shown that freshly isolated decidual NK cells from mid-gestation viable and resorbing embryos had no marked cytolytic activities against fetal cells or Yac-1 targets (Linnemeyer and Pollack, 1991). Hence, it could be that NK cells exert their cytolytic activities indirectly by activating other effector cells, such as decidual macrophages, through the production of cytokines such as IFN–γ and TNF–α.

Decidual macrophages

Macrophages are the ultimate effector cells of many immune responses. They are called monocytes in the circulating blood, and by many names when they become residents of extravascular tissues (Nielsen et al., 1994). Macrophages function to eliminate microorganisms and other antigens from tissues by two mechanisms. First, a direct mechanism where macrophages engulf the microbe or antigen and degrade it in cytoplasmic vesicles or by secreting a variety of enzymes (Gottschall et al., 1995; Deng et al., 1995), oxygen (Rugtveit et al., 1995; Mattana et al., 1995) and nitrogen radicals (Liew et al., 1991; Nathan and Hibbs, Jr. 1991). The second mechanism involves processing and presenting the antigen to the immune systems. This is

known to initiate the immune response through the activation and recruiting of T-cells and B-cells (Ellis et al., 1991; Walker and Sun, 1991).

The activation of macrophages is not a single step process. Macrophage activation is measured by the ability of a macrophage to perform a particular function measured by a specific assay. For example, a macrophage can kill a certain microorganism in one assay, but it can not kill another in a different assay. It appears that macrophage activation depends on the expression of a number of different genes. Which of the genes is being expressed after a particular stimulus would dictate the nature of the macrophage response. Macrophages are activated by a variety of stimuli including lipopolysaccharide (LPS) (Landmann et al., 1991), lipoproteins (Cheung et al., 1991), TNF–α (Brugger et al., 1991; Kopelovich, 1991), and IFN–γ (Fan et al., 1991; Jungi et al., 1991). Interferon–γ is considered to be the major macrophage activation factor.

The role of macrophages in mammalian reproduction has been studied extensively both in humans as well as in mice. Macrophages are present in both virgin and pregnant uterus (De and Wood, 1990; Hume et al., 1984). Nonetheless, the number of decidual macrophages increased markedly during pregnancy when compared to that in virgin mice (Stewart and Mitchell, 1991). This is probably due to the local

inflammatory reaction associated with implantation. It has been shown that the number of macrophages was also increased in pseudopregnant mice suggesting that the process of decidualization, and not the implantation of the embryo itself was responsible for the recruitment of macrophages to the uterine site (Choudhuri and Wood, 1992).

Studies concerning the presence of macrophages at implantation sites during normal pregnancy have been controversial. Studies with F4/80, a specific marker for macrophages, have shown that mature macrophages were scarce in the deciduum of normal murine pregnancy (Redline et al., 1990). This contradicted other studies which showed that F4/80 positive cells were numerous in normal murine decidua (Pollard et al., 1991, Brandon, 1995). The basis for this dispute is poorly understood since in both studies the same antibody was used.

The role of decidual macrophages in normal pregnancy is not well defined. It has been assumed that decidual macrophages were needed to eliminate bacteria and parasitic infections at the fetomaternal interface and to form the decidua in which the embryo implants (Hunt, 1990). Decidual macrophages have been shown to produce enzymes and cytokines that were believed to be critical for normal reproduction. These includes colony stimulating factor (CSF-1) which is known to induce the growth of trophoblast cells which express c-fms, the CSF-1 receptors (Pampfer et al., 1991). Others cytokines such as transforming

growth factor–β (TGF–β) produced by macrophages has also been shown to suppress the maternal anti-paternal immune responses allowing embryo survival (Lea and Clark, 1991).

Recent studies have indicated that macrophages are the primary effector cells associated with early embryo loss. Using the CBA/J X DBA/2 model, it has been shown that the number of macrophages were significantly higher in resorbing when compared to non-resorbing embryos (Duclos et al., 1995; Duclos et al., 1994). In these studies three macrophage markers were used and the results were consistent in all the experiments performed. Further studies done on normal human decidua showed the presence of macrophages which expressed surface MHC class II antigens (Hunt, 1989). In vivo depletion experiments have further shown that mice injected with antibodies to the Mac-1, a macrophage marker, showed a significant increase in embryo survival rate as compared to mice injected with the isotype-matched control monoclonal antibody (Duclos, personal communication).

In spontaneous abortion, the state of decidual macrophage activation is not fully elucidated. More interestingly, it is not clear what factors are responsible for macrophage activation and how macrophages mediate effector functions against the growing embryo. The thesis will present results addressing these questions in chapters 2, 3, and 4.

Soluble Effectors

Interferons-α (IFN–α),-β (IFN–β), and -γ (IFN–γ)

Interferon–α and IFN–β are type I interferons. They are inflammatory cytokines that are expressed in infected cells as an early response to viral infections (Shalaby et al., 1985; Stiehm et al., 1982). Interferon–α is exclusively produced by cells of lymphoid origin including leukocytes and macrophages (Williams, 1991), while IFN–β is produced by fibroblasts and epithelial cells (Ezaki et al., 1991).

The role of type I interferons in embryo resorption have been the subject of increased attention since pregnant mice treated with Poly I:C, double stranded RNA, which induce type I interferon production, significantly augmented embryo loss when injected into pregnant female mice (Lin et al., 1991; Pyo et al., 1991). Further, type I interferons induce NK cell activation, suggesting that type I interferons are associated with embryo resorption through enhancement of NK cell activities.

Interferon–γ is a type II or lymphocyte interferon which is one of the most important immunoregulatory molecules to have been described in the last 30 years. T-cells and NK cells are the two cell types that account for most of the IFN–γ production (Benveniste and Benos, 1995; Young and Hardy, 1995). Interferon–γ was first described in the

literature for its anti-viral and anti-tumor activities. Interferon–γ has been shown to be the major cytokine responsible for the activation of macrophage resistance to microbes and cancer (Mossman et al., 1995; Biron, 1994). Interferon–γ activates macrophages to produce a variety of cytokines and factors such as TNF–α, oxygen and nitrogen radicals that have been shown to be extremely efficient in eliminating microorganisms (Guidotti et al., 1994; Noronha et al., 1993). Interferon–γ enhances the expression of MHC class II molecules on antigen presenting cells including macrophages and B-cells (Tanaka, 1994). In mice, Interferon–γ is known to be produced by TH0, and TH1 cell populations (Murphy et al., 1996). Interestingly, it has been shown that IFN–γ suppressed the production of TH2 cytokines thus enhancing TH1 mediated cellular immunity (Xu et al., 1996).

Interferon–γ is believed to be involved with viral clearance in normal pregnancy. However, if IFN-γ was actively produced by decidual NK cells, a major effector cell population associated with early embryo loss, it was proposed that the initiation of decidual IFN–γ production might be critical in determining the success or failure of a pregnancy. Studies involving site specific localization of IFN-γ expression at the implantation sites are still lacking. However, the presence of IFN-γ receptor in human and mouse macrophages, endometrial cells and

trophoblast has been reported by many investihgtors (Chen et al., 1994c; Paulesu et al., 1994)

It has been reported that IFN-γ was associated with early embryo loss. Exogenous IFN-γ injected into pregnant mice significantly augmented the incidence of embryo loss (Chaouat et al., 1990). It has been further shown that a significant increase in systemic IFN–γ production was observed in mice that had a high incidence of embryo loss and not in those with a low incidence of embryo loss (Krishnan et al., 1996b). However, the mechanisms by which IFN–γ mediate embryo resorption are not fully understood, and further studies of the local production and expression have not been addressed.

Interferon-γ exerts its effect through binding to a specific receptor. Interferon-γ receptor belongs to the Type II cytokine receptors, which also include receptors for type I interferons. The extracellular domain of the IFN-γ receptor comprises two immunoglobulin domains that contain a set of four spaced cysteins (Miyajima et al., 1992; Kishimoto et al., 1994). The mouse IFN-γ receptor gene has been cloned and sequenced by different investigators (Kumar et al., 1989; Gray et al., 1989), and the results have shown that these receptors are ubiquitously expressed in many tissues and cell lines. Expression of the IFN-γ receptor gene in mouse uterus and placenta have been studied at the mRNA as well as the protein levels. The results have shown that the levels of expression

of the IFN-γ receptor in the placenta and uterus increased with gestational age and were selective to certain cell populations including specific placental cells, macrophages and NK-like cells (Chen et al., 1994a; Chen et al., 1994b). These results concluded that IFN-γ might play a primary role in the normal development of the embryo in murine pregnancy. However, these studies have not investigated the functional importance of these receptors. Recently, the IFN-γ receptor knockout mouse has been generated. The reported data showed that these mice were healthy and showed normal reproductive performance (Huang et al., 1993) indicating that IFN-γ regulation through IFN-γ receptors was not necessary to assure successful pregnancies.

Chapter 3 addresses the role of IFN–γ in spontaneous abortion in IFN-γ knockout mice and suggests mechanisms by which IFN–γ mediates embryo loss.

Tumor necrosis factor–α (TNF–α)

Although many cells are capable of producing TNF-α, activated macrophages are considered the major source for its production. TNF–α is a multifunctional cytokine which has been first identified by killing tumor cells (Sugarman et al., 1985; Nedwin et al., 1985b). Now, it is known that TNF–α is the principal mediator of the host response against gram negative bacteria and other infectious organisms (Hachiya et al.,

1995; Degre and Bukholm, 1995). Further, TNF–α can be a growth and differentiating factor in other tissues. Tumor necrosis factor-α is a trimer and is expressed as a an integral transmembrane precursor of 26 kd. Proteolytic cleavages release the 17 kd mature protein into the medium (Kriegler et al., 1988; Scuderi, 1989). The genes that code for TNF-α are located within the MHC genes on chromosome 6 in humans and 17 in mice (Nedwin et al., 1985a; Nedospasov et al., 1986).

The induction of TNF-α by different cell populations is dependent on the stimulus used. For example, tissue macrophages and monocytes are primarily stimulated with LPS (Aggarwal et al., 1985a; Beutler et al., 1985). On the other hand, B-cells, T-cells and NK cells produce TNF-α to different stimuli such as phorbol diester, phytohemagglutinin, and concanvalin A (Christmas and Meager, 1990; Cuturi et al., 1987).

In the context of pregnancy outcome, TNF–α had been shown to be associated with embryo loss. Mice injected with TNF–α showed a high incidence of embryo loss (Chaouat et al., 1990). On the other hand, treatment with pentoxyfilline, an inhibitor of TNF–α production, or anti-TNF–α antibodies augmented embryo survival (Roberts et al., 1990; Baines and Gendron, 1993).

Tumor necrosis factor-α mediates its effect through binding to specific receptors. Two TNF-α receptors have been identified, the TNFR1 (55 kd) and TNFR2 (75 kd). Both of these receptors have been shown to

be expressed in many tissues (Aggarwal et al., 1985b). The two receptors belong to the TNF-like receptor family which also contains Neuron growth factor receptor, Fas/Apo antigens, CD40 and many others (Stamenkovic et al., 1989). Interestingly, these receptors have been shown to induce programmed cell death or apoptosis in many cells and tissues (Cleveland and Ihle, 1995). In general, TNFR1 has been shown to be responsible for most of TNF-α cytotoxic effects, while TNFR2 was more involved in cell proliferation (Jacobsen et al., 1994).

The expression of TNF receptors has been studied in both human and murine implantation sites. The results have shown that TNFR1 and TNFR2 were expressed in most of the tissues tested. TNF receptors transcripts were found in the uterine epithelium and trophoblasts. The results further showed an increased selective expressions of the TNF receptors in some cell populations with gestational age, suggesting that TNF-α might be involved in regulating the growth of growing embryos (Yelavarthi and Hunt, 1993). Conversely, knockout mice that were deficient in TNFR1 and TNFR2 showed no signs of abnormal or impaired pregnancy parameters (Pfeffer et al., 1993; Erickson et al., 1994). Recently, in vitro studies has shown that TNFR1, but not TNFR2 was responsible for inducing apoptosis in primary villous trophoblasts suggesting that TNF-α production might be deleterious to the growing fetus (Yui et al., 1996).

Besides TNF-α production, activated macrophages are also known to produce a number of cytotoxic molecules such as nitric oxide. The property of nitric oxide has been reviewed extensively in chapters 2, 3, and 4. I refer the reader to these chapters for references.

The role of TNF–α and nitric oxide in early embryo loss has been discussed in chapters 2, 3, and 4.

Immunotrophic and Immunosuppressive Soluble Factors

Immunotrophism is a hypothesis in which it is proposed that the maternal immune response developed against fetal cells leads to the production and release of cytokines that can serve as positive growth factor for trophoblasts (Lala et al., 1983c). This hypothesis was supported by the observation that immunostimulation of the pregnant female mice with paternal or third party leukocytes enhanced embryo survival. Some of these immunotrophic factors have been identified and they include cytokines such as IL-3, GM-CSF, and CSF-1 (Chatterjee-Hasrouni and Lala, 1982; Lala and Kearns, 1985; Lala et al., 1983b). The role of these cytokines in determining pregnancy outcome has been studied in a mouse model of high embryo loss. The results showed that administration of any of these cytokines into pregnant mice augmented embryo survival (Hunt et al., 1988). Furthermore, depleting T-cells during pregnancy, which is the only uterine source of the IL-3 and GM-

CSF, compromised pregnancy outcome (Athanassakis et al., 1987). However, these results have been disputed by data derived from mice with genetically engineered gene knockouts and natural mutations in T-cells and MHC antigens. It has been shown that SCID, nude, Beige and MHC deficient mice were all able to reproduce normally (Clark et al., 1989). These results do not deny the importance of immunotrophic cytokines in pregnancy success, but rather suggest that a source different that T-cells may be responsible for their production.

Immunosuppression

The theory of immunosuppression in pregnancy has been the bases of many mechanisms to explain the survival of allogeneic fetus. However, as mentioned previously, there was no agreement on the identification and characterization of the immunosuppressive cells at the fetomaternal interface. Interestingly, it was observed that cytokines which had been known to suppress cell mediated cytotoxcicity were associated with embryo survival. Two of these cytokines, TGF-β and IL-10, have been studied in the murine model of early embryo loss . It was shown that the expression of both cytokines were increased in healthy embryos and decreased in resorbing embryos. Administration of IL-10 to gravid mice with high embryo loss significantly augmented embryo

survival (Chaouat et al., 1995). On the other hand, anti-IL-10 antibodies had the opposite effects and lead to increased embryo resorption.

Conclusion and Prospective

Many hypothesis have been postulated to explain the mechanisms that leads to spontaneous abortion or early embryo loss. Although, some of these postulates account for genetic , anatomic, endocrine, and other mechanisms, the immunological etiology of early embryo loss is receiving increased attention. Most of our knowledge on embryo loss is based on observation derived from human and murine models of early embryo loss. It is impossible to identify one mechanism that could explain all the reported observations and results on repeated abortion.

One of the most consistent observations is that the TH1/TH2 cytokine balance is very critical to pregnancy outcome. It was postulated that the success of pregnancy is normally maintained by a dominance of TH2 over TH1 cytokines. This review undisputedly showed that TH1 cytokines endangered embryo survival and that TH2 cytokines enhanced embryo survival. Due to the overall paucity of decidual T-cells, the presence of TH1 and TH2 cells in the implantation sites, on the other hand, is controversial. Hence which cell populations, other than T-cells, are responsible for maintaining the cytokine balance is currently unclear. Studies by some have shown that γ/δ T-cells were present at the

implantation sites, and that these cells could mediate an immune response through the non-traditional recognition of heat shock proteins and production of regulatory cytokines. Other non-immune cell populations, such as trophoblast cells, natural killer cells and macrophages might be involved. Whether it is an immune or a non-immune initiating mechanism, the activation of the immune effector arm has been shown to be extremely critical to pregnancy outcome.

In this study, I will explore the innate and non-specific immune effector mechanisms involved in early embryo loss. I will present evidence that activated macrophages are responsible for most of the damage in early abortion through the production of nitric oxide. I will show that TH1 cytokines such as IFN–γ and TNF–α are the cytokines responsible for macrophage activation and leading consequently to embryo loss.

References

Aggarwal, B.B., Eessalu, T.E., and Hass, P.E. (1985a). Characterization of receptors for human tumour necrosis factor and their regulation by gamma-interferon. Nature *318*, 665-667.

Aggarwal, B.B., Kohr, W.J., Hass, P.E., Moffat, B., Spencer, S.A., Henzel, W.J., Bringman, T.S., Nedwin, G.E., Goeddel, D.V., and Harkins, R.N. (1985b). Human tumor necrosis factor. Production, purification, and characterization. Journal of Biological Chemistry *260*, 2345-2354.

Ahlfors, C.E., Goetzman, B.W., Halsted, C.C., Sherman, M.P., and Wennberg, R.P. (1977). Neonatal listeriosis. American Journal of Diseases of Children *131*, 405-408.

Allison, J.P. and Havran, W.L. (1991). The immunobiology of T cells with invariant gamma delta antigen receptors. [Review]. Annual Review of Immunology *9*, 679-705.

Allison, J.P. and Raulet, D.H. (1990). The immunobiology of gamma delta+ T cells. [Review]. Seminars in Immunology *2*, 59-65.

Anonymous, (1994). World wide collaborative study and meta-analysis on allogenic leukocyte immunotherapy for recurrent sponatneous abortion. Recurrent Miscarriage Immunotherapy Trialist group. Am. J. Reprod. Immunol. *32,* 55-72.

Athanassakis, I., Bleackley, R.C., Paetkau, V., Guilbert, L., Barr, P.J., and Wegmann, T.G. (1987). The immunostimulatory effect of T cell and T cell lymphokines on murine fetally derived cells. J. Immunol. *138*, 37-44.

Athanassakis-Vassiliadis, I., Galanopoulos, V.K., Grigoriou, M., and Papamatheakis, J. (1990). Induction of Class-II MHC Antigen Expression on the Murine Placenta by 5-Azacytidine Correlates with Fetal Abortion. Cell. Immunol. *128*, 438-449.

Baines, M.G. and Gendron, R.L. (1993). Natural and experimental animal models of reproductive failure. In Immunology of Pregnancy. G. Chaouat, ed. (Boca Raton: CRC Press, Inc.), pp. 173-203.

Baines, M.G., Duclos, A.J., and Haddad, E.K. (1997) Decidual leukocytes infiltration and activation of macrophages leads to early embryo loss. J. Reprod. Immunol. In press.

Beer, A.E., Quebbeman, J.F., Ayers, J.W., and Haines, R.F. (1981). Major histocompatibility complex antigens, maternal and paternal immune responses, and chronic habitual abortions in humans. American Journal of Obstetrics & Gynecology *141*, 987-999.

Benveniste, E.N. and Benos, D.J. (1995). TNF-alpha- and IFN-gamma-mediated signal transduction pathways: effects on glial cell gene expression and function. [Review]. FASEB Journal *9*, 1577-1584.

Bernard, O., Scheid, M.P., Ripoche, M.A., and Bennett, D. (1978). Immunological studies of mouse decidual cells. I. Membrane markers of decidual cells in the days after implantation. J. Exp. Med. *148*, 580-591.

Berthou, C., Marolleau, J.P., Lafaurie, C., Soulie, A., Dal Cortivo, L., Bourge, J.F., Benbunan, M., and Sasportes, M. (1995). Granzyme B and perforin lytic proteins are expressed in CD34+ peripheral blood progenitor cells mobilized by chemotherapy and granulocyte colony-stimulating factor. Blood *86*, 3500-3506.

Beutler, B., Mahoney, J., Le Trang, N., Pekala, P., and Cerami, A. (1985). Purification of cachectin, a lipoprotein lipase-suppressing hormone secreted by endotoxin-induced RAW 264.7 cells. J. Exp. Med. *161*, 984-995.

Biron, C.A. (1994). Cytokines in the generation of immune responses to, and resolution of, virus infection. [Review]. Current Opinion in Immunology *6*, 530-538.

Bischof, P. and Martelli, M. (1992). Current topic: proteolysis in the penetration phase of the implantation process. [Review]. Placenta *13*, 17-24.

Blank, M., Cohen, J., Toder, V., and Shoenfeld, Y. (1991) Induction of anti-phospholipid syndrome in naive mice with mouse lupus monoclonal and human polyclonal anti-cardiolipin antibodies. Proc. Natl. Acad. Sci. U.S.A. *88*: 3069-73.

Bluestone, J.A., Pardoll, D., Sharrow, S.O., and Fowlkes, B.J. (1987). Characterization of murine thymocytes with CD3-associated T-cell receptor structures. Nature *326*, 82-84.

Bochan, M.R., Goebel, W.S., and Brahmi, Z. (1995). Stably transfected antisense granzyme B and perforin constructs inhibit human granule-mediated lytic ability. Cell. Immunol. *164*, 234-239.

Born, W., Happ, M.P., Dallas, A., Reardon, C., Kubo, R., Shinnick, T., Brennan, P., and O'Brien, R. (1990). Recognition of heat shock proteins and gamma delta cell function. [Review]. Immunology Today *11*, 40-43.

Bosma, G.C., Custer, R.P., and Bosma, M.J. (1983). A severe combined immunodeficiency mutation in the mouse. Nature *301*, 527-530.

Brandon, J.M. (1995) Macrophage distribution in decidual tissue from early implantation to the periparturient period in mice as defined by the macrophage differentiation antigens F4/80, macrosialin and the type 3 complement receptor. J. Reprod. Fert. *103*: 9-16.

Brugger, W., Reinhardt, D., Galanos, C., and Andreesen, R. (1991). Inhibition of in vitro differentiation of human monocytes to macrophages by lipopolysaccharides (LPS): phenotypic and functional analysis. International Immunology *3*, 221-227.

Bulmer, D., Peel, S., and Stewart, I. (1987). The metrial gland. [Review]. Cell Differentiation *20*, 77-86.

Vassiliadou, N., and Bulmer, J.N. (1996) Quantitative analysis of T lymphocyte subsets in pregnant and nonpregnant human endometrium. Biol. Reprod. *55*, 1017-1022.

Chaouat, G., Kiger, N., and Wegmann, T.G. (1983). Vaccination against spontaneous abortion in mice. J. Reprod. Immunol. *5*, 389-392.

Chaouat, G., Menu, E., Clark, D.A., Dy, M., Minkowski, M., and Wegmann, T.G. (1990). Control of fetal survival in CBA x DBA/2 mice by lymphokine therapy. J. Reprod. Fert. *89*, 447-458.

Chaouat, G., Assal Meliani, A., Martal, J., Raghupathy, R., Elliot, J., Mosmann, T.R., and Wegmann, T.G. 1995. IL-10 prevents naturally occurring fetal loss in the CBA x DBA/2 mating combination, and local defect in IL-10 production in this abortion-prone combination is corrected by in vivo injection of IFN-tau. J.Immunol. *154*: 4261-4268.

Chatelain, R., Varkila, K., and Coffman, R.L. (1992). IL-4 induces a Th2 response in Leishmania major-infected mice. J. Immunol. *148*, 1182-1187.

Chatterjee-Hasrouni, S. and Lala, P.K. (1982). Localization of paternal H-2K antigens on murine trophoblast cells in vivo. J. Exp. Med. *155*, 1679-1689.

Chen, H.L., Kamath, R., Pace, J.L., Russell, S.W., and Hunt, J.S. (1994a). Expression of the interferon-gamma receptor gene in mouse placentas is related to stage of gestation and is restricted to specific subpopulations of trophoblast cells. Placenta *15*, 109-121.

Chen, H.L., Kamath, R., Pace, J.L., Russell, S.W., and Hunt, J.S. (1994b). Gestation-related expression of the interferon-gamma receptor gene in mouse uterine and embryonic hematopoietic cells. J. Leuk. Biol. *55*, 617-625.

Chen, H.L., Kamath, R., Pace, J.L., Russell, S.W., and Hunt, J.S. (1994c). Gestation-related expression of the interferon-gamma receptor gene in mouse uterine and embryonic hematopoietic cells. J. Leuk. Biol. *55*, 617-625.

Cheung, S.C., Chattopadhyay, S.K., Hartley, J.W., Morse, H.C., 3d, and Pitha, P.M. (1991). Aberrant expression of cytokine genes in peritoneal macrophages from mice infected with LP-BM5 MuLV, a murine model of AIDS. J. Immunol. *146*, 121-127.

Choudhuri, R. and Wood, G.W. (1992). Leukocyte distribution in the pseudopregnant mouse uterus. Am. J. Reprod. Immunol. *27*, 69-76.

Christmas, S.E. and Meager, A. (1990). Production of interferon-gamma and tumour necrosis factor-alpha by human T-cell clones expressing different forms of the gamma delta receptor. Immunology *71*, 486-492.

Clark, D.A., McDermott, M.R., and Szewczuk, M.R. (1980). Impairment of host-versus-graft reaction in pregnant mice. II. Selective suppression of cytotoxic T-cell generation correlates with soluble suppressor activity and with successful allogeneic pregnancy. Cell. Immunol. *52*, 106-118.

Clark, D.A., Chaput, A., and Tutton, D. (1986). Active suppression of host-vs-graft reaction in pregnant mice. VII. Spontaneous abortion of allogeneic CBA/J x DBA/2 fetuses in the uterus of CBA/J mice

correlates with deficient non-T suppressor cell activity. J. Immunol. *136*, 1668-1675.

Clark, D.A. and McDermott, M.R. (1978). Impairment of host vs graft reaction in pregnant mice. I. Suppression of cytotoxic T cell generation in lymph nodes draining the uterus. J. Immunol. *121*, 1389-1393.

Clark, D.A., Vince, G., Flanders, K.C., Hirte, H., and Starkey, P. (1994) CD56+ lymphoid cells in human first trimester pregnancy decidua as a source of novel transforming growth factor-beta 2-related immunosuppressive factors. Human Reproduction. *9*: 2270-7.

Clark, D.A., Banwatt, D.K., Manuel, J., Fulop, G and Croy, B.A. 1989, SCID mice in reproductive Immunology. Curr.top.Micro.Immunol. *152*227-233.

Clarke, G.N. and Baker, H.W. (1993). Lack of association between sperm antibodies and recurrent spontaneous abortion [see comments]. Fertility & Sterility *59*, 463-464.

Cleveland, J.L. and Ihle, J.N. (1995). Contenders in FasL/TNF death signaling. [Review]. Cell *81*, 479-482.

Cohen, B.L., Orn, A., Gronvik, K.O., Gidlund, M., Wigzell, H., and Murgita, R.A. (1986). Suppression by alpha-fetoprotein of murine natural killer cell activity stimulated in vitro and in vivo by interferon and interleukin 2. Scand. J. Immunol. *23*, 211-223.

Coulam, C.B. (1992). Immunologic tests in the evaluation of reproductive disorders: a critical review. American Journal of Obstetrics & Gynecology *167*, 1844-1851.

Cox, G.W., Mathieson, B.J., Giardina, S.L., and Varesio, L. (1990). Characterization of IL-2 receptor expression and function on murine macrophages. J. Immunol. *145*, 1719-1726.

Cox, G.W., Chattopadhyay, U., Oppenheim, J.J., and Varesio, L. (1991). IL-4 inhibits the costimulatory activity of IL-2 or picolinic acid but not of lipopolysaccharide on IFN-gamma-treated macrophages. J. Immunol. *147*, 3809-3814.

Crepeau, M.A. and Croy, B.A. (1988). Evidence that specific cellular immunity cannot account for death of Mus caroli embryos transferred to

Mus musculus with severe combined immune deficiency disease. Transplantation *45*, 1104-1110.

Croy, B.A., Gambel, P., Rossant, J., and Wegmann, T.G. (1985a). Characterization of murine decidual natural killer (NK) cells and their relevance to the success of pregnancy. Cell. Immunol. *93*, 315-326.

Croy, B.A., Rossant, J., and Clark, D.A. (1985b). Effects of alterations in the immunocompetent status of Mus musculus females on the survival of transferred Mus caroli embryos. Journal of Reproduction & Fertility *74*, 479-489.

Croy, B.A., Guilbert, L.J., Browne, M.A., Gough, N.M., Stinchcomb, D.T., Reed, N., and Wegmann, T.G. (1991). Characterization of cytokine production by the metrial gland and granulated metrial gland cells. J. Reprod. Immunol. *19*, 149-166.

Croy, B.A. and Kiso, Y. (1993). Granulated metrial gland cells: a natural killer cell subset of the pregnant murine uterus. [Review]. Microscopy Research & Technique *25*, 189-200.

Croy, B.A. (1994) Granulated metrial gland cells: hypotheses concerning

possible functions during murine gestation. J.Reprod.Immunol. *27*: 85-94.

Cuturi, M.C., Murphy, M., Costa-Giomi, M.P., Weinmann, R., Perussia, B., and Trinchieri, G. (1987). Independent regulation of tumor necrosis factor and lymphotoxin production by human peripheral blood lymphocytes. J. Exp. Med. *165*, 1581-1594.

de Fougerolles, A.R. and Baines, M.G. (1987). Modulation of the natural killer cell activity in pregnant mice alters the spontaneous abortion rate. J. Reprod. Immunol. *11*, 147-153.

De, M. and Wood, G.W. (1990). Influence of oestrogen and progesterone on macrophage distribution in the mouse uterus. Journal of Endocrinology *126*, 417-424.

Degre, M. and Bukholm, G. (1995). Orally administered interferon-gamma but not tumor necrosis factor-alpha suppress infection with Salmonella typhimurium in a mouse model. Journal of Biological Regulators & Homeostatic Agents *9*, 15-20.

Deleze, M., Alarcon-Segovia, D., Oria, C.V., Sanchez-Guerrero, J., Fernandez-Dominguez, L., Gomez-Pacheco, L., and Ponce, d.L. (1989). Hemocytopenia in systemic lupus erythematosus. Relationship to antiphospholipid antibodies [published erratum appears in J Rheumatol 1989 Nov;16(11):1523]. Journal of Rheumatology *16*, 926-930.

Deng, J., Rudick, V., and Dory, L. (1995). Lysosomal degradation and sorting of apolipoprotein E in macrophages. Journal of Lipid Research *36*, 2129-2140.

Doherty, P.C., Allan, W., Eichelberger, M., and Carding, S.R. (1991). Heat-shock proteins and the gamma delta T cell response in virus infections: implications for autoimmunity. [Review]. Springer Seminars in Immunopathology *13*, 11-24.

Dosch, H.M., Lee, J.W., Gelfand, E.W., and Falk, J.A. (1978). Severe combined immunodeficiency disease: a model of T-cell dysfunction. Clinical & Experimental Immunology *34*, 260-267.

Duclos, A.J., Pomerantz, D.K., and Baines, M.G. (1994). Relationship between decidual leukocyte infiltration and spontaneous abortion in a murine model of early fetal resorption. Cell. Immunol. *159*, 184-193.

Duclos, A.J., Haddad, E.K., and Baines, M.G. (1995). Presence of activated macrophages in a murine model of early embryo loss. Am. J. Reprod. Immunol. *33*, 354-366.

Duclos, A.J., Haddad, E.K., Chalifour, L.E., and Baines, M.G. (1996). Embryo infiltration by maternal macrophages is associated with selective expression of proto-oncogenes in a murine model of spontaneous abortion. Biology of Reproduction *54*, 1088-1095.

El-Roeiy, A. and Shoenfeld, Y. (1985). Autoimmunity and pregnancy. [Review]. American Journal of Reproductive Immunology & Microbiology *9*, 25-32.

Ellis, J., Chain, B.M., Davies, D.H., Ibrahim, M.A., Katz, D.R., Kaye, P.M., and Lightstone, E. (1991). Antigen presentation by dendritic cells provides optimal stimulation for the production of interleukin (IL) 2, IL 4 and interferon-gamma by allogeneic T cells. European Journal of Immunology *21*, 2803-2809.

Ellis, S. (1990). HLA G: at the interface. Am. J. Reprod. Immunol. *23*, 84-86.

Ellis, S.A., Palmer, M.S., and McMichael, A.J. (1990). Human trophoblast and the choriocarcinoma cell line BeWo express a truncated HLA Class I molecule. J. Immunol. *144*, 731-735.

Erickson, S.L., de Sauvage, F.J., Kikly, K., Carver-Moore, K., Pitts-Meek, S., Gillett, N., Sheehan, K.C., Schreiber, R.D., Goeddel, D.V., and Moore, M.W. (1994). Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. Nature *372*, 560-563.

Evron, S., Brenner, T., and Abramsky, O. (1984). Suppressive effect of pregnancy on the development of experimental allergic encephalomyelitis in rabbits. Am. J. Reprod. Immunol. *5*, 109-113.

Ezaki, K., Suzuki, M., Miyazaki, H., Maruyama, F., Sobue, R., Okamoto, M., Matsui, T., Ino, T., Shimizu, K., Hirano, M., and et a (1991). Mixed lymphocyte-autologous tumor cell reaction in hematological malignancies--effect of interferon-beta and correlation with the expression of MHC class I antigen on tumor cells. Journal of Interferon Research *11*, 305-310.

Fan, S., Fehr, H.G., and Adams, D. (1991). Activation of macrophages for ADCC in vitro: effects of IL-4, TNF, interferons-alpha/beta, interferon-gamma, and GM-CSF. Cell. Immunol. *135*, 78-87.

Faulk, W.P. and Temple, A. (1976). Distribution of B2-microglobulin in chorionic villi of human placentae. Nature *262*, 799-802.

Ferrick, D.A., Schrenzel, M.D., Mulvania, T., Hsieh, B., Ferlin, W.G., and Lepper, H. (1995). Differential production of interferon-gamma and interleukin-4 in response to Th1- and Th2-stimulating pathogens by gamma delta T cells in vivo. Nature *373*, 255-257.

Ferrini, S., Cambiaggi, A., Meazza, R., Sforzini, S., Marciano, S., Mingari, M.C., and Moretta, L. (1994). T cell clones expressing the natural killer cell-related p58 receptor molecule display heterogeneity in phenotypic properties and p58 function. European Journal of Immunology *24*, 2294-2298.

Fitzgerald, T.J. (1992). The Th1/Th2-like switch in syphilitic infection: is it detrimental?. [Review]. Infection & Immunity *60*, 3475-3479.

Fleischer, B. (1991). Stimulation of human T cells by microbial 'superantigens'. [Review]. Immunologic Research *10*, 349-355.

Fowlkes, B.J. and Pardoll, D.M. (1989). Molecular and cellular events of T cell development. [Review]. Advances in Immunology *44*, 207-264.

Gambel, P., Croy, B.A., Moore, W.D., Hunziker, R.D., Wegmann, T.G., and Rossant, J. (1985). Characterization of immune effector cells present in early murine decidua. Cell. Immunol. *93*, 303-314.

Gendron, R.L., Farookhi, R., and Baines, M.G. (1990). Resorption of CBA/J x DBA/2 mouse conceptuses in CBA/J uteri correlates with failure of the feto-placental unit to suppress natural killer cell activity. Journal of Reproduction & Fertility *89*, 277-284.

Gendron, R.L. and Baines, M.G. (1989). Morphometric analysis of the histology of spontaneous fetal resorption in a murine pregnancy. Placenta *10*, 309-318.

Gottschall, P.E., Yu, X., and Bing, B. (1995). Increased production of gelatinase B (matrix metalloproteinase-9) and interleukin-6 by activated rat microglia in culture. Journal of Neuroscience Research *42*, 335-342.

Gray, P.W., Leong, S., Fennie, E.H., Farrar, M.A., Pingel, J.T., Fernandez-Luna, J., and Schreiber, R.D. (1989). Cloning and expression of the cDNA for the murine interferon gamma receptor. Proceedings of the National Academy of Sciences of the United States of America *86*, 8497-8501.

Griffin, J.P., Harshan, K.V., Born, W.K., and Orme, I.M. (1991). Kinetics of accumulation of gamma delta receptor-bearing T lymphocytes in mice infected with live mycobacteria. Infection & Immunity *59*, 4263-4265.

Grusby, M.J., Johnson, R.S., Papaioannou, V.E., and Glimcher, L.H. (1991). Depletion of CD4+ T cells in major histocompatibility complex class II-deficient mice. Science *253*, 1417-1420.

Guidotti, L.G., Ando, K., Hobbs, M.V., Ishikawa, T., Runkel, L., Schreiber, R.D., and Chisari, F.V. (1994). Cytotoxic T lymphocytes inhibit hepatitis B virus gene expression by a noncytolytic mechanism in

transgenic mice. Proceedings of the National Academy of Sciences of the United States of America *91*, 3764-3768.

Guimond, M.J., Wang, B., Fujita, J., Terhorst, C., and Croy, B.A. (1996). Pregnancy-associated uterine granulated metrial gland cells in mutant and transgenic mice. Am. J. Reprod. Immunol. *35*, 501-509.

Haas, G.G., Jr., Kubota, K., Quebbeman, J.F., Jijon, A., Menge, A.C., and Beer, A.E. (1986). Circulating antisperm antibodies in recurrently aborting women. Fertility & Sterility *45*, 209-215.

Haas, W., Pereira, P., and Tonegawa, S. (1993). Gamma/delta cells. [Review]. Annual Review of Immunology *11*, 637-685.

Hachiya, O., Takeda, Y., Miyata, H., Watanabe, H., Yamashita, T., and Sendo, F. (1995). Inhibition by bacterial lipopolysaccharide of spontaneous and TNF-alpha-induced human neutrophil apoptosis in vitro. Microbiology & Immunology *39*, 715-723.

Hara, T., Ohashi, S., Yamashita, Y., Abe, T., Hisaeda, H., Himeno, K., Good, R.A., and Takeshita, K. (1996). Human Vdelta2+ gamma delta T-cell tolerance to foreign antigens of Toxoplasma gondii. Proceedings of

the National Academy of Sciences of the United States of America *93*, 5136-5140.

Harris, E.N., Gharavi, A.E., and Hughes, G.R. (1985). Anti-phospholipid antibodies. [Review]. Clinics in Rheumatic Diseases *11*, 591-609.

Harris, E.N., Asherson, R.A., and Hughes, G.R. (1988). Antiphospholipid antibodies--autoantibodies with a difference. [Review]. Annual Review of Medicine *39*, 261-271.

Havran, W.L. and Allison, J.P. (1988). Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. Nature *335*, 443-445.

Head, J.R., Kresge, C.K., Young, J.D., and Hiserodt, J.C. (1994). NKR-P1+ cells in the rat uterus: granulated metrial gland cells are of the natural killer cell lineage. Biology of Reproduction *51*, 509-523.

Hess, J., Ladel, C., Miko, D., and Kaufmann, S.H. (1996). Salmonella typhimurium aroA- infection in gene-targeted immunodeficient mice: major role of CD4+ TCR-alpha beta cells and IFN-gamma in bacterial

clearance independent of intracellular location. J. Immunol. *156*, 3321-3326.

Heyborne, K.D., Cranfill, R.L., Carding, S.R., Born, W.K., and O'Brien, R.L. (1992). Characterization of gamma delta T lymphocytes at the maternal-fetal interface. J. Immunol. *149*, 2872-2878.

Hill, J.A., Polgar, K., and Anderson, D.J. (1995). T-helper 1-type immunity to trophoblast in women with recurrent spontaneous abortion. JAMA *273*, 1933-1936.

Hill, J.A. (1991). The rationale for leukocyte immunization in women with recurrent spontaneous abortion. Fact or fiction? In Collogue INSERM Cellular and Molecular Biology of the Maternal Fetal Relationship. G. Chaouat and J. Mowbray, ed. (John Libbey Eurotext), 263-269.

Hooper, D.C., Pagida, E., and Murgita, R.A. (1982). Suppression of primary and secondary autologous mixed lymphocyte reactions by murine alphafetoprotein. Oncodevelopmental Biology & Medicine *3*, 151-160.

Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, R.M., and Aguet, M. (1993). Immune response in mice that lack the interferon-gamma receptor [see comments]. Science *259*, 1742-1745.

Hume, D.A., Halpin, D., Charlton, H., and Gordon, S. (1984). The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80: macrophages of endocrine organs. Proceedings of the National Academy of Sciences of the United States of America *81*, 4174-4177.

Hunt, J.S., Manning, L.S., Mitchell, D., Selanders, J.R., and Wood, G.W. (1985). Localization and characterization of macrophages in murine uterus. J. Leuk. Biol. *38*, 255-265.

Hunt, J.S., Fishback, J.L., Andrews, G.K., and Wood, G.W. (1988). Expression of classI MHC by trophoblast cells: Analysis by in situ hybridization. J. Immunol. *140*, 1293-1299.

Hunt, J.S. (1989). Macrophages in human uteroplacental tissues: a review. [Review]. Am. J. Reprod. Immunol. *21*, 119-122.

Hunt, J.S. (1990). Current topic: the role of macrophages in the uterine response to pregnancy. [Review]. Placenta *11*, 467-475.

Ikuta, S., Kirby, J.A., Shenton, B.K., Givan, A.L., and Lennard, T.W. (1991). Human endothelial cells: effect of TNF-alpha on peripheral blood mononuclear cell adhesion. Immunology *73*, 71-76.

Itohara, S., Farr, A.G., Lafaille, J.J., Bonneville, M., Takagaki, Y., Haas, W., and Tonegawa, S. (1990). Homing of a gamma delta thymocyte subset with homogeneous T-cell receptors to mucosal epithelia. Nature *343*, 754-757.

Iwashima, M., Green, A., Bonyhadi, M., Davis, M.M., Allison, J.P., and Chien, Y.H. (1991). Expression of a fetal gamma delta T-cell receptor in adult mice triggers a non-MHC-linked form of selective depletion. International Immunology *3*, 385-393.

Jacobsen, F.W., Rothe, M., Rusten, L., Goeddel, D.V., Smeland, E.B., Veiby, O.P., Slordal, L., and Jacobsen, S.E. (1994). Role of the 75-kDa tumor necrosis factor receptor: inhibition of early hematopoiesis.

Proceedings of the National Academy of Sciences of the United States of America *91*, 10695-10699.

Jager, S., Kremer, J., and De Wilde-Janssen, I.W. (1984). Are sperm immobilizing antibodies in cervical mucus an explanation for a poor postcoital test? Am. J. Reprod. Immunol. *5*, 56-60.

Janeway, C.A., Jr., Carding, S., Jones, B., Murray, J., Portoles, P., Rasmussen, R., Rojo, J., Saizawa, K., West, J., and Bottomly, K. (1988). CD4+ T cells: specificity and function. [Review]. Immunol. Rev. *101*, 39-80.

Jungers, P., Liote, F., Dautzenberg, M.D., Gazengel, C., Dougados, M., Tron, F., and Bach, J.F. (1984). Lupus anticoagulant and thrombosis in systemic lupus erythematosus [letter]. Lancet *1*, 574-575.

Jungi, T.W., Brcic, M., Leutwyler, C., Pfister, H., and Spycher, M.O. (1991). Interferon-gamma treatment impairs Fc receptor type II-mediated phagocytosis of human macrophages by a post-receptor-binding mechanism. Immunology *74*, 439-445.

Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, K.J., Podack, E.R., Zinkernagel, R.M., and Hengartner, H. (1994). Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice [see comments]. Nature *369*, 31-37.

Kane, K.P. and Clark, W.R. (1986). Class I MHC Antigens in the Generation and Expression of Promiscuous Cytotoxic Cell Function. J. Immunol. *137*, 3080-3087.

Kaufmann, S.H., Schoel, B., van Embden, J.D., Koga, T., Wand-Wurttenberger, A., Munk, M.E., and Steinhoff, U. (1991). Heat-shock protein 60: implications for pathogenesis of and protection against bacterial infections. [Review]. Immunol. Rev. *121*, 67-90.

Khong, T.Y. (1989). Pathology of intrauterine growth retardation [published erratum appears in Am J Reprod Immunol 1990 Aug;23(4):129]. [Review]. Am. J. Reprod. Immunol. *21*, 132-136.

Kiger, N., Chaouat, G., Kolb, J.P., Wegmann, T.G., and Guenet, J.L. (1985). Immunogenetic Studies of Spontaneous Abortion in Mice: Preimmunization of Females with Allogeneic Cells. J. Immunol. *134*, 2966-2970.

Kinsky, R., Delage, G., Rosin, N., Thang, M.N., Hoffmann, M., and Chaouat, G. (1990). A murine model of NK cell mediated resorption. Am. J. Reprod. Immunol. *23*, 73-77.

Kishimoto, T., Taga, T., and Akira, S. (1994). Cytokine signal transduction. [Review]. Cell *76*, 253-262.

Koning, F., Kruisbeek, A.M., Maloy, W.L., Marusic-Galesic, S., Pardoll, D.M., Shevach, E.M., Stingl, G., Valas, R., Yokoyama, W.M., and Coligan, J.E. (1988a). T cell receptor gamma/delta chain diversity. J. Exp. Med. *167*, 676-681.

Koning, F., Yokoyama, W.M., Maloy, W.L., Stingl, G., McConnell, T.J., Cohen, D.I., Shevach, E.M., and Coligan, J.E. (1988b). Expression of C gamma 4 T cell receptors and lack of isotype exclusion by dendritic epidermal T cell lines. J. Immunol. *141*, 2057-2062.

Kopelovich, L. (1991). Conversion of human fibroblasts to tissue macrophages by the Snyder-Theilen feline sarcoma virus (ST:FeSV) is associated with the de-novo expression of IL-1 alpha, IL-1 beta, IFN-

alpha, TNF-alpha, GM-CSF, and CD4. European Cytokine Network *2*, 99-106.

Kovatts, S., Librach, C., Fisch, P., Main, E.K., Sondel, P. M., Fisher, S.J., and DeMars, R. (1991). The role of non-classical MHC class I on human trophoblast, in Biologie Cellulaire et Moleculaire de la Relation Materno Fetale, Chaouat, G. And Mowbray, J., Eds., Editions INSERM/John Lebbey, Paris, pp: 13-21.

Kriegler, M., Perez, C., DeFay, K., Albert, I., and Lu, S.D. (1988). A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. Cell *53*, 45-53.

Krishnan, L., Guilbert, L.J., Russell, A.S., Wegmann, T.G., Mosmann, T.R., and Belosevic, M. (1996a). Pregnancy impairs resistance of C57BL/6 mice to Leishmania major infection and causes decreased antigen-specific IFN-gamma response and increased production of T helper 2 cytokines. J. Immunol. *156*, 644-652.

Krishnan, L., Guilbert, L.J., Wegmann, T.G., Belosevic, M., and Mosmann, T.R. (1996b). T helper 1 response against Leishmania major in pregnant C57BL/6 mice increases implantation failure and fetal

resorptions. Correlation with increased IFN-gamma and TNF and reduced IL-10 production by placental cells. J. Immunol. *156*, 653-662.

Kuchroo, V.K., Das, M.P., Brown, J.A., Ranger, A.M., Zamvil, S.S., Sobel, R.A., Weiner, H.L., Nabavi, N., and Glimcher, L.H. (1995). B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. Cell *80*, 707-718.

Kumar, C.S., Muthukumaran, G., Frost, L.J., Noe, M., Ahn, Y.H., Mariano, T.M., and Pestka, S. (1989). Molecular characterization of the murine interferon gamma receptor cDNA. Journal of Biological Chemistry *264*, 17939-17946.

Kwak, J.Y.H., Gilman-Sachs, A., Beaman, K.D., and Beer, A.E. (1992). Autoantibodies in women with primary recurrent spontaneous abortion of unknown etiology. J. Reprod. Immunol. *22*, 15-31.

Kwak, J.Y.H., Barini, R., Gilman-Sachs, A., Beaman, K.D., and Beer, A.E. (1994). Down-regulation of maternal antiphospholipid antibodies during early pregnancy and pregnancy outcome. American Journal of Obstetrics & Gynecology *171*, 239-246.

Lachapelle MH. Miron P. Hemmings R. Roy DC. (1996). Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion. Altered profile and pregnancy outcome. J. Immunol. *156*, 4027-4034.

Ladel, C.H., Blum, C., and Kaufmann, S.H. (1996). Control of natural killer cell-mediated innate resistance against the intracellular pathogen Listeria monocytogenes by gamma/delta T lymphocytes. Infection & Immunity *64*, 1744-1749.

Lala, P.K., Chatterjee-Hasrouni, S., Kearns, M., Montgomery, B., and Colavincenzo, V. (1983a). Immunobiology of the feto-maternal interface. [Review]. Immunol. Rev. *75*, 87-116.

Lala, P.K., Chatterjee-Hasrouni, S., Kearns, M., Montgomery, B., and Colavincenzo, V. (1983b). Immunobiology of the feto-maternal interface. Immunol. Rev. *75*, 87-116.

Lala, P.K., Chatterjee-Hasrouni, S., and Montgomery, B. (1983c). MHC antigens on murine and human trophoblast cells. Transp. Proc. *15*, 883-886.

Lala, P.K. and Kearns, M. (1985). Immunobiology of the decidual tissue. Contrib. Gynec. Obstet. *14*, 1-15.

Landmann, R., Ludwig, C., Obrist, R., and Obrecht, J.P. (1991). Effect of cytokines and lipopolysaccharide on CD14 antigen expression in human monocytes and macrophages. Journal of Cellular Biochemistry *47*, 317-329.

Lea, R.G. and Clark, D.A. (1991). Macrophages and migratory cells in endometrium relevant to implantation. [Review]. Baillieres Clinical Obstetrics & Gynaecology *5*, 25-59.

Lichtenstein, M.R. (1942). Tuberculin reaction in Tuberculosis during pregnancy. American Review of Tuberculosis. *46*, 89-92.

Liew, F.Y., Li, Y., Moss, D., Parkinson, C., Rogers, M.V., and Moncada, S. (1991). Resistance to Leishmania major infection correlates with the induction of nitric oxide synthase in murine macrophages. European Journal of Immunology *21*, 3009-3014.

Lin, H.Y., Davis, P.J., and Thacore, H.R. (1991). Production of human interferon-beta by Sendai virus and poly(rI).poly(rC): inhibition by neomycin. Journal of Interferon Research *11*, 365-369.

Lin, P.Y., Joag, S.V., Young, J.D., Chang, Y.S., Soong, Y.K., and Kuo, T.T. (1991). Expression of perforin by natural killer cells within first trimester endometrium in humans. Biology of Reproduction *45*, 698-703.

Lindemann, R.A. (1991). The regulatory effects of monocytes on human natural killer cells activated by lipopolysaccharides. Journal of Periodontal Research *26*, 486-490.

Linnemeyer, P.A. and Pollack, S.B. (1991). Murine granulated metrial gland cells at uterine implantation sites are natural killer lineage cells. J. Immunol. *147*, 2530-2535.

Lockshin, M.D. (1985). Lupus pregnancy. [Review]. Clinics in Rheumatic Diseases *11*, 611-632.

Lubbe, W.F. and Liggins, G.C. (1985). Lupus anticoagulant and pregnancy. [Review]. American Journal of Obstetrics & Gynecology *153*, 322-327.

MacDougall, J.R., Croy, B.A., Chapeau, C., and Clark, D.A. (1990). Demonstration of a splenic cytotoxic effector cell in mice of genotype SCID/SCID.BG/BG. Cell. Immunol. *130*, 106-117.

Madden, D.R. (1995). The three-dimensional structure of peptide-MHC complexes. [Review]. Annual Review of Immunology *13*, 587-622.

Marshall, R.J. and Jones, D.B. (1988). An immunohistochemical study of lymphoid tissue in human endometrium. International Journal of Gynecological Pathology *7*, 225-235.

Matis, L.A. and Bluestone, J.A. (1991). Specificity of gamma delta receptor bearing T cells. [Review]. Seminars in Immunology *3*, 75-80.

Mattana, J., Margiloff, L., and Singhal, P.C. (1995). Oxidation of mesangial matrix using a mixed function oxidase system augments adhesion of macrophages: possible role of macrophage scavenger

receptors. Biochemical & Biophysical Research Communications *212*, 63-69.

McBride, W.H., Economou, J.S., Nayersina, R., Comora, S., and Essner, R. (1990). Influences of interleukins 2 and 4 on tumor necrosis factor production by murine mononuclear phagocytes. Cancer Research *50*, 2949-2952.

McIntyre, J.A. and Faulk, W.P. (1983). Recurrent spontaneous abortion in human pregnancy: results of immunogenetical, cellular, and humoral studies. Am. J. Reprod. Immunol. *4*, 165-170.

McLauchlin, J. (1990). Human listeriosis in Britain, 1967-85, a summary of 722 cases. 2. Listeriosis in non-pregnant individuals, a changing pattern of infection and seasonal incidence. Epidemiology & Infection *104*, 191-201.

Meeusen, E., Fox, A., Brandon, M., and Lee, C.S. (1993). Activation of uterine intraepithelial gamma delta T cell receptor-positive lymphocytes during pregnancy. European Journal of Immunology *23*, 1112-1117.

Melnick, M., Jaskoll, T., and Slavkin, H.C. (1981). Corticosteroid-induced cleft palate in mice and H-2 haplotype: maternal and embryonic effects. Immunogenetics *13*, 443-450.

Menge, A.C. (1970). Immune reactions and infertility. [Review]. Journal of Reproduction & Fertility - Supplement *10*, 171-186.

Mincheva-Nilsson, L., Hammarstrom, S., and Hammarstrom, M.L. (1992). Human decidual leukocytes from early pregnancy contain high numbers of gamma delta+ cells and show selective down-regulation of alloreactivity. J. Immunol. *149*, 2203-2211.

Mincheva-Nilsson, L., Baranov, V., Yeung, M.M., Hammarstrom, S., and Hammarstrom, M.L. (1994). Immunomorphologic studies of human decidua-associated lymphoid cells in normal early pregnancy. J. Immunol. *152*, 2020-2032.

Makino, T., Hara, T., Oka, C., Toyoshima, K., Sugi, T., Iwasaki, K., and Umeuchi, M. (1992) Survey of 1120 Japanese women with a history of recurrent spontaneous abortions. Eur. J. Obst. Gyn. Reprod. Biol. *44*:123-30.

Miyajima, A., Kitamura, T., Harada, N., Yokota, T., and Arai, K. (1992). Cytokine receptors and signal transduction. [Review]. Annual Review of Immunology *10*, 295-331.

Mizoguchi, A., Mizoguchi, E., Chiba, C., Spiekermann, G.M., Tonegawa, S., Nagler-Anderson, C., and Bhan, A.K. (1996). Cytokine imbalance and autoantibody production in T cell receptor-alpha mutant mice with inflammatory bowel disease. J. Exp. Med. *183*, 847-856.

Montesoro, E., Bulgarini, D., Care, A., Masciulli, R., Giannella, G., Mariani, G., Samoggia, P., Salvo, G., Habetswallner, D., Testa, U., and et a (1991). Long-term culture growth of CD4-CD8- lymphocytes exhibiting elevated non-MHC-restricted cytotoxic activity. Journal of Biological Regulators & Homeostatic Agents *5*, 10-18.

Moore, M.P., Carter, N.P., and Redman, C.W. (1983). Lymphocyte subsets in normal and pre-eclamptic pregnancies. British Journal of Obstetrics & Gynaecology *90*, 326-331.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., and Coffman, R.L. (1986). Two types of murine helper T cell clone. I.

Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. *136*, 2348-2357.

Mosmann, T.R. and Coffman, R.L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. [Review]. Annual Review of Immunology *7*, 145-173.

Mossman, K., Upton, C., Buller, R.M., and McFadden, G. (1995). Species specificity of ectromelia virus and vaccinia virus interferon-gamma binding proteins. Virology *208*, 762-769.

Murgita, R.A. (1976). The immunosuppressive role of AFP furing pregnancy. Scand. J. Immunol. *5*, 1003-1012.

Murphy, E., Shibuya, K., Hosken, N., Openshaw, P., Maino, V., Davis, K., Murphy, K., and O'Garra, A. (1996). Reversibility of T helper 1 and 2 populations is lost after long-term stimulation. J. Exp. Med. *183*, 901-913.

Murphy, J.W. (1985). Natural cell-mediated resistance against Cryptococcus neoformans: a possible role for natural killer (NK) cells. [Review]. Current Topics in Medical Mycology *1*, 135-154.

Murray, D.L., Imagawa, D.T., Okada, D.M., and St Geme, J.W., Jr. (1979). Antibody response to monovalent A/New Jersey/8/76 influenza vaccine in pregnant women. Journal of Clinical Microbiology *10*, 184-187.

Nandi, D. and Allison, J.P. (1991). Phenotypic analysis and gamma delta-T cell receptor repertoire of murine T cells associated with the vaginal epithelium. J. Immunol. *147*, 1773-1778.

Narumi, S., Finke, J.H., and Hamilton, T.A. (1990). Interferon gamma and interleukin 2 synergize to induce selective monokine expression in murine peritoneal macrophages. Journal of Biological Chemistry *265*, 7036-7041.

Nathan, C.F. and Hibbs, J.B., Jr. (1991). Role of nitric oxide synthesis in macrophage antimicrobial activity. [Review]. Current Opinion in Immunology *3*, 65-70.

Naume, B., Shalaby, R., Lesslauer, W., and Espevik, T. (1991). Involvement of the 55- and 75-kDa tumor necrosis factor receptors in

the generation of lymphokine-activated killer cell activity and proliferation of natural killer cells. J. Immunol. *146*, 3045-3048.

Naz, R.K., Ellaurie, M., Phillips, T.M., and Hall, J. (1990). Antisperm antibodies in human immunodeficiency virus infection: effects on fertilization and embryonic development. Biology of Reproduction *42*, 859-868.

Nedospasov, S.A., Hirt, B., Shakhov, A.N., Dobrynin, V.N., Kawashima, E., Accolla, R.S., and Jongeneel, C.V. (1986). The genes for tumor necrosis factor (TNF-alpha) and lymphotoxin (TNF-beta) are tandemly arranged on chromosome 17 of the mouse. Nucleic Acids. Res. *14*, 7713-7725.

Nedwin, G.E., Naylor, S.L., Sakaguchi, A.Y., Smith, D., Jarrett-Nedwin, J., Pennica, D., Goeddel, D.V., and Gray, P.W. (1985a). Human lymphotoxin and tumor necrosis factor genes: structure, homology and chromosomal localization. Nucleic Acids. Res. *13*, 6361-6373.

Nedwin, G.E., Svedersky, L.P., Bringman, T.S., Palladino, M.A., Jr., and Goeddel, D.V. (1985b). Effect of interleukin 2, interferon-gamma, and

mitogens on the production of tumor necrosis factors alpha and beta. J. Immunol. *135*, 2492-2497.

Nielsen, B.W., Mukaida, N., Matsushima, K., and Kasahara, T. (1994). Macrophages as producers of chemotactic proinflammatory cytokines. [Review]. Immunology Series *60*, 131-142.

Nilssen, D.E., Muller, F., Oktedalen, O., Froland, S.S., Fausa, O., Halstensen, T.S., and Brandtzaeg, P. (1996). Intraepithelial gamma/delta T cells in duodenal mucosa are related to the immune state and survival time in AIDS. Journal of Virology *70*, 3545-3550.

Nobel, C. (1984). Malimplantaion, a cause of failure after IVF and GIFT. Am. J. Rep. Immunol. *6*, 56-57.

Nonaka, K., Miura, T., and Peter, K. (1994). Recent fertility decline in Dariusleut Hutterities: an extension of Eaton and Mayer's Hutterite fertility study. Human Biology *66*, 411-420.

Noronha, A., Toscas, A., and Jensen, M.A. (1993). Interferon beta decreases T cell activation and interferon gamma production in multiple sclerosis. Journal of Neuroimmunology *46*, 145-153.

O'Brien, R.L. and Born, W. (1991). Heat shock proteins as antigens for gamma delta T cells. [Review]. Seminars in Immunology *3*, 81-87.

Ober, C.L., Martin, A.O., Simpson, J.L., Hauck, W.W., Amos, D.B., Kostyu, D.D., Fotino, M., and Allen, F.H., Jr. (1983). Shared HLA antigens and reproductive performance among Hutterites. American Journal of Human Genetics *35*, 994-1004.

Ober, C.L., Hauck, W.W., Kostyu, D.D., O'Brien, E., Elias, S., Simpson, J.L., and Martin, A.O. (1985). Adverse effects of human leukocyte antigen-DR sharing on fertility: a cohort study in a human isolate. Fertility & Sterility *44*, 227-232.

Oksenhendler, E., Bourbigot, B., Desbazeille, F., Droz, D., Choquenet, C., Girot, R., and Jungers, P. (1984). Recurrent hematuria in 4 white patients with sickle cell trait. Journal of Urology *132*, 1201-1203.

Ortaldo, J.R. (1991). Human cytotoxic effector cells: definition and analysis of activity. [Review]. Allergologia et Immunopathologia *19*, 145-156.

Pampfer, S., Arceci, R.J., and Pollard, J.W. (1991). Role of colony stimulating factor-1 (CSF-1) and other lympho-hematopoietic growth factors in mouse pre-implantation development. [Review]. Bioessays *13*, 535-540.

Pardoll, D.M., Fowlkes, B.J., Bluestone, J.A., Kruisbeek, A., Maloy, W.L., Coligan, J.E., and Schwartz, R.H. (1987). Differential expression of two distinct T-cell receptors during thymocyte development. Nature *326*, 79-81.

Parr, E.L., Young, L.H., Parr, M.B., and Young, J.D. (1990). Granulated metrial gland cells of pregnant mouse uterus are natural killer-like cells that contain perforin and serine esterases. J. Immunol. *145*, 2365-2372.

Paulesu, L., Romagnoli, R., Cintorino, M., Ricci, M.G., and Garotta, G. (1994). First trimester human trophoblast expresses both interferon-gamma and interferon-gamma-receptor. J. Reprod. Immunol. *27*, 37-48.

Peel, S. and Adam, E. (1991). The killing of rat placental cells by rat and mouse granulated metrial gland cells in vitro. Placenta *12*, 161-171.

Peel, S. and Stewart, I. (1984). The differentiation of granulated metrial gland cells in chimeric mice and the effect of uterine shielding during irradiation. Journal of Anatomy *139*, 593-598.

Pfeffer, K., Matsuyama, T., Kundig, T.M., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P.S., Kronke, M., and Mak, T.W. (1993). Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell *73*, 457-467.

Pollard, J.W., Hunt, J.S., Wiktor-Jedrzejczak, W., and Stanley, E.R. (1991). A pregnancy defect in the osteopetrotic (op/op) mouse demonstrates the requirement for CSF-1 in female fertility. Developmental Biology *148*, 273-283.

Pyo, S., Gangemi, J.D., Ghaffar, A., and Mayer, E.P. (1991). Poly I:C-induced anti-herpes simplex virus type 1 activity in inflammatory macrophages is mediated by induction of interferon-beta. J. Leuk. Biol. *50*, 479-487.

Raulet, D.H. (1989). The structure, function, and molecular genetics of the gamma/delta T cell receptor. [Review]. Annual Review of Immunology *7*, 175-207.

Redline, R.W., McKay, D.B., Vazquez, M.A., Papaioannou, V.E., and Lu, C.Y. (1990). Macrophage functions are regulated by the substratum of murine decidual stromal cells. Journal of Clinical Investigation *85*, 1951-1958.

Redline, R.W. and Lu, C.Y. (1987). Role of local immunosuppression in murine fetoplacental listeriosis. Journal of Clinical Investigation *79*, 1234-1241.

Redline, R.W. and Lu, C.Y. (1988). Specific defects in the anti-listerial immune response in discrete regions of the murine uterus and placenta account for susceptibility to infection. J. Immunol. *140*: 3947-55.

Redman, C.W.G. (1993). The placenta, pre-eclampsia and chronic villitis. In The human placenta. C.W.G. Redman, I.L. Sargent, and P.M. Starkey, eds. (Oxford: Blackwell Scientific Publications), pp. 433-467.

Roberts, R.M., Farin, C.E., and Cross, J.C. (1990). Trophoblast proteins and maternal recognition of pregnancy. [Review]. Oxford Rev. Reprod. Immunol. *12*, 147-180.

Rocklin, R.E., Kitzmiller, J.L., Carpenter, C.B., Garovoy, M.R., and David, J.R. (1976). Maternal-fetal relation. Absence of an immunologic blocking factor from the serum of women with chronic abortions. New England Journal of Medicine *295*, 1209-1213.

Rocklin, R.E., Kitzmiller, J.L., and Garvoy, M.R. (1982). Maternal-fetal relation. II. Further characterization of an immunologic blocking factor that develops during pregnancy. Clinical Immunology & Immunopathology *22*, 305-315.

Roder, J. and Duwe, A. (1979). The beige mutation in the mouse selectively impairs natural killer cell function. Nature *278*, 451-453.

Romagnani, S. (1994). Lymphokine production by human T cells in disease states. [Review]. Annual Review of Immunology *12*, 227-257.

Roth, C., Kourilsky, P., and Ojcius, D.M. (1994). Ly-49-independent inhibition of natural killer cell-mediated cytotoxicity by a soluble major

histocompatibility complex class I molecule. European Journal of Immunology *24*, 2110-2114.

Rugtveit, J., Haraldsen, G., Hogasen, A.K., Bakka, A., Brandtzaeg, P., and Scott, H. (1995). Respiratory burst of intestinal macrophages in inflammatory bowel disease is mainly caused by CD14+L1+ monocyte derived cells. Gut *37*, 367-373.

Sammaritano, L.R., Gharavi, A.E., and Lockshin, M.D. (1990). Antiphospholipid antibody syndrome: immunologic and clinical aspects. [Review]. Seminars in Arthritis & Rheumatism *20*, 81-96.

Sanders, S.K., Giblin, P.A., and Kavathas, P. (1991). Cell-cell adhesion mediated by CD8 and human histocompatibility leukocyte antigen G, a nonclassical major histocompatibility complex class 1 molecule on cytotrophoblasts. J. Exp. Med. *174*, 737-740.

Scott, J.R., Rote, N.S., and Branch, D.W. (1987). Immunologic aspects of recurrent abortion and fetal death. [Review]. Obstet. Gynecol. *70*, 645-656.

Scuderi, P. (1989). Suppression of human leukocyte tumor necrosis factor secretion by the serine protease inhibitor p-toluenesulfonyl-L-arginine methyl ester (TAME). J. Immunol. *143*, 168-173.

Seder, R.A. and Paul, W.E. (1994). Acquisition of lymphokine-producing phenotype by CD4+ T cells. [Review]. Annual Review of Immunology *12*, 635-673.

Shalaby, M.R., Hamilton, E.B., Benninger, A.H., and Marafino, B.J., Jr. (1985). In vivo antiviral activity of recombinant murine gamma interferon. Journal of Interferon Research *5*, 339-345.

Shukla, H., Swaroop, A., Srivastava, R., and Weissman, S.M. (1990). The mRNA of a human class I gene HLA G/HLA 6.0 exhibits a restricted pattern of expression. Nucleic Acids. Res. *18*, 2189

Sridama, V., Pacini, F., Yang, S.L., Moawad, A., Reilly, M., and DeGroot, L.J. (1982). Decreased levels of helper T cells: a possible cause of immunodeficiency in pregnancy. New England Journal of Medicine *307*, 352-356.

Stamenkovic, I., Clark, E.A., and Seed, B. (1989). A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. EMBO Journal *8*, 1403-1410.

Starkey, P.M., Sargent, I.L., and Redman, C.W. (1988). Cell populations in human early pregnancy decidua: characterization and isolation of large granular lymphocytes by flow cytometry. Immunology *65*, 129-134.

Stewart, I.J. (1994). Granulated metrial gland cells--not part of the natural killer cell lineage?. [Review]. J. Reprod. Immunol. *26*, 1-15.

Stewart, I.J. and Mitchell, B.S. (1991). The distribution of uterine macrophages in virgin and early pregnant mice. Journal of Anatomy *179*, 183-196.

Sthoeger, Z.M., Tartakovsky, B., Bentwich, Z., and Mozes, E. (1993). Monoclonal anticardiolipin antibodies derived from mice with experimental lupus erythematosus: characterization and the induction of a secondary antiphospholipid syndrome. Journal of Clinical Immunology *13*, 127-138.

Stiehm, E.R., Kronenberg, L.H., Rosenblatt, H.M., Bryson, Y., and Merigan, T.C. (1982). UCLA conference. Interferon: immunobiology and clinical significance. [Review]. Annals of Internal Medicine *96*, 80-93.

Stingl, G., Gunter, K.C., Tschachler, E., Yamada, H., Lechler, R.I., Yokoyama, W.M., Steiner, G., Germain, R.N., and Shevach, E.M. (1987). Thy-1+ dendritic epidermal cells belong to the T-cell lineage. Proceedings of the National Academy of Sciences of the United States of America *84*, 2430-2434.

Stray-Pedersen, B. and Stray-Pedersen, S. (1984). Etiologic factors and subsequent reproductive performance in 195 couples with a prior history of habitual abortion. American Journal of Obstetrics & Gynecology *148*, 140-146.

Street, N.E. and Mosmann, T.R. (1991). Functional diversity of T lymphocytes due to secretion of different cytokine patterns. [Review]. FASEB Journal *5*, 171-177.

Sugarman, B.J., Aggarwal, B.B., Hass, P.E., Figari, I.S., Palladino, M.A., Jr., and Shepard, H.M. (1985). Recombinant human tumor necrosis

factor-alpha: effects on proliferation of normal and transformed cells in vitro. Science *230*, 943-945.

Sunderland, C.A., Redman, C.W.G., and Stirrat, G.M. (1981). HLA A,B,C antigens are expressed on nonvillous trophoblast of the early human placenta. J. Immunol. *127*, 2614-2615.

Tanaka, K. (1994). Role of proteasomes modified by interferon-gamma in antigen processing. [Review]. J. Leuk. Biol. *56*, 571-575.

Toder, V., Nebel, L., and Gleicher, N. (1984). Studies of natural killer cells in pregnancy. I. Analysis at the single cell level. Journal of Clinical & Laboratory Immunology *14*, 123-127.

Toder, V., Strassburger, D., Carp, H., and Irlin, I. (1991). Mouse model for the treatment of immune pregnancy loss. [Review]. Am. J. Reprod. Immunol. *26*, 42-46.

Tomasi, T.B., Jr., Dattwyler, R.J., Murgita, R.A., and Keller, R.H. (1975). Immunosuppression by alpha-fetoprotein. [Review]. Transactions of the Association of American Physicians *88*, 293-299.

Trinchieri, G. (1989). Biology of natural killer cells. [Review]. Advances in Immunology *47*, 187-376.

Tschachler, E., Schuler, G., Hutterer, J., Leibl, H., Wolff, K., and Stingl, G. (1983). Expression of Thy-1 antigen by murine epidermal cells. Journal of Investigative Dermatology *81*, 282-285.

Tsuyuguchi, I., Kawasumi, H., Ueta, C., Yano, I., and Kishimoto, S. (1991). Increase of T-cell receptor gamma/delta-bearing T cells in cord blood of newborn babies obtained by in vitro stimulation with mycobacterial cord factor. Infection & Immunity *59*, 3053-3059.

Tupper, C. and Weil, K.J. (1962). The problem of spontaneous abortion. American Journal of Obstetrics & Gynecology *83*, 423-427.

van den Broek, M.F., Kagi, D., Zinkernagel, R.M., and Hengartner, H. (1995). Perforin dependence of natural killer cell-mediated tumor control in vivo. European Journal of Immunology *25*, 3514-3516.

Vidovic, D., Roglic, M., McKune, K., Guerder, S., MacKay, C., and Dembic, Z. (1989). Qa-1 restricted recognition of foreign antigen by a gamma delta T-cell hybridoma. Nature *340*, 646-650.

Villacres-Eriksson, M. (1995). Antigen presentation by naive macrophages, dendritic cells and B cells to primed T lymphocytes and their cytokine production following exposure to immunostimulating complexes. Clinical & Experimental Immunology *102*, 46-52.

Walker, W.S. and Sun, D. (1991). Constitutive antigen presentation by mouse splenic macrophages is restricted to the progeny of a distinct progenitor population. Cell. Immunol. *133*, 342-351.

Wegmann, T.G., Lin, H., Guilbert, L., and Mosmann, T.R. (1993). Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? [see comments]. [Review]. Immunology Today *14*, 353-356.

Weinberg, E.D. (1984). Pregnancy-associated depression of cell-mediated immunity. [Review]. Reviews of Infectious Diseases *6*, 814-831.

Williams, B.R. (1991). Signal transduction and transcriptional regulation of interferon-alpha-stimulated genes. [Review]. Journal of Interferon Research *11*, 207-213.

Xu, H., DiIulio, N.A., and Fairchild, R.L. (1996). T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon gamma-producing (Tc1) effector CD8+ T cells and interleukin (Il) 4/Il-10-producing (Th2) negative regulatory CD4+ T cells. J. Exp. Med. *183*, 1001-1012.

Yan, Y., Liang, P., Wang, L., Miao, S., and Koide, S.S. (1990). Inhibition of zygote formation and embryo development in mice immunized with monoclonal antisperm antibodies. Proceedings of the Chinese Academy of Medical Sciences & the Peking Union Medical College *5*, 9-12.

Yelavarthi, K.K. and Hunt, J.S. (1993). Analysis of p60 and p80 tumor necrosis factor-alpha receptor messenger RNA and protein in human placentas. Am. J. Pathol. *143*, 1131-1141.

Yewdell, J.W. and Bennink, J.R. (1992). Cell biology of antigen processing and presentation to major histocompatibility complex class I

molecule-restricted T lymphocytes. [Review]. Advances in Immunology *52*, 1-123.

Young, H.A. and Hardy, K.J. (1995). Role of interferon-gamma in immune cell regulation. [Review]. J. Leuk. Biol. *58*, 373-381.

Yui, J., Hemmings, D., Garcialloret, M., and Guilbert, L.J. (1996). Expression of the human p55 and p75 tumor necrosis factor receptors in primary villous trophoblasts and their role in cytotoxic signal transduction. Biology of Reproduction *55*, 400-409.

Zijlstra, M., Bix, M., Simister, N.E., Loring, J.M., Raulet, D.H., and Jaenisch, R. (1990). Beta 2-microglobulin deficient mice lack CD4-8+ cytolytic T cells. Nature *344*, 742-746.

Preface to chapter 2

We have previously shown that both NK cells and macrophages were associated with early embryo loss. In this study, we studied the state of decidual macrophage activation at the implantation sites of the high and low resorption matings. We further investigated the role of nitric oxide, a cytotoxic molecule produced by activated macrophages, on embryo survival.

Early Embryo Loss Is Associated with Local Production of Nitric Oxide by Decidual Mononuclear Cells

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Summary

In early embryo loss, the fetus may be considered to be an allograft and, therefore, may be rejected by maternal immunocytes. However, the cytotoxic mechanisms involved are still poorly understood. We have previously shown the involvement of natural killer (NK) cells and mononuclear cells expressing Mac-1 (CD11b) and F4/80 in resorbing compared to nonresorbing embryos. In this study, the role of nitric oxide (NO) in the mechanism of early embryo loss was studied. Pregnant CBA/J females mated with DBA/2 males (20–30% early embryo loss) and CD1 females mated with CD1 males (5–10% early embryo loss) were studied on days 8, 10, and 12 of gestation. Cells from the implantation sites of individual embryos were tested for the production of nitrite and nitrate with or without in vitro challenge with lipopolysaccharide (LPS) to determine whether decidual macrophages were primed in situ. On day 12 of gestation, when resorption was clearly visible, resorbing embryos showed more than a fivefold increase in both basal- and LPS-induced nitrite and nitrate production compared to nonresorbing embryos in both mouse strains tested, indicating that the decidual mononuclear cells were primed. Furthermore, more than 20% of CBA/J embryos showed a significant nitrate release on days 8 and 10 of gestation before any signs of embryo cytopathology. This percentage corresponded to the spontaneous resorption rate seen in CBA/J female × DBA/2 male matings. Similarly, 4% of the embryos from pregnant CD1 mice on days 8 and 12 of gestation produced a significant amount of nitrate, which again correlated with the low incidence of resorption observed in these mice. Using immunohistochemistry, the presence of inducible nitric oxide synthase (iNOS) was detected at implantation sites. Furthermore, decidual cells positive for both iNOS and the macrophage marker Mac-1 were demonstrated in implantation sites by double immunostaining. This strongly suggests that decidual macrophages could be the cellular source of NO production. Aminoguanidine, a selective inhibitor of the iNOS, inhibited the in vitro production of nitric oxide by cells isolated from individual implantation sites, and more strikingly, significantly reduced early embryo losses in CBA/J females mated by DBA/2 males when given orally or parenterally to the gravid females starting on day 6 of gestation. In addition, aminoguanidine-treated pregnant mice showed a significant increase in average litter size when the pregnancies were allowed to proceed to term. The results presented strongly suggested a role for NO as an effector molecule in mediating early embryo loss and showed that the in situ activation of decidual macrophages was an early event preceding spontaneous abortion.

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The mechanisms of early embryo loss observed in humans and many other animal species are not yet fully understood. Most of our knowledge regarding the mechanism of early fetal resorption has been obtained by studying the cell biology of resorption prone matings of CBA/J X DBA/2 mice. The natural resorption rate in CBA/J females when mated by DBA/2 males is 20%-25% (1). This could be further increased to 60% of the litter if the females were injected with Poly I:C, a synthetic double stranded RNA molecule, capable of inducing interferons which activate innate resistance (2). The mechanism of enhancement of early embryo loss by poly I:C showed a similar time course and cytopathology to that observed in the untreated CBA/J females mated with DBA/2 males (3,4). By using this experimental model, we have previously shown that NK cells were associated with early fetal rejection. NK cells heavily infiltrated some, but not all embryos at day 8 of gestation (5). Furthermore, injecting pregnant CBA/J mice with anti-Asialo-GM1, a rabbit anti-NK cell antiserum, significantly reduced the incidence of resorption (6). Recently, we reported the association of maternal macrophages with early embryo loss (3). Implantation sites from potentially resorbing embryos showed a significant increase in decidual Mac-1, and F4/80 positive cells compared to non-resorbing embryos. In addition, other researchers have shown that normal embryos show significant numbers of leukocytes in the decidual and placental tissues (7-9). Taken together, these results suggested a role for macrophages and NK cells in mediating early fetal rejection. However, these studies did not address the cytolytic effector mechanism involved and the state of activation of the infiltrated leukocytes.

The involvement of cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-2 (IL-2) in the mechanism of fetal rejection have been reported (10). Interleukin-2 induces the production of IFN-γ by NK cells (11). Interferon-γ secreted by NK cells primes macrophages for TNF-α and nitric oxide (NO) production (12-14). In fact, these cytokines were shown to increase the frequency of resorption in CBA/J female X DBA/2 male matings (10). However, direct evidence of embryotoxic effects of these cytokines in mediating embryo loss is still lacking.

In this study, we investigated the role of macrophage activation in early embryo loss as determined by the release of nitric oxide, a macrophage derived cytotoxic molecule, synthesized by an enzyme called nitric oxide synthase (NOS). Nitric oxide is a short-lived mediator which can be induced in a variety of cell types and produces many physiologic and metabolic changes in target tissues and cells (15). At least three isotypes of the NOS enzyme have been identified, including two constitutive isotypes (cNOS), found in endothelial and neuronal cells, and an inducible isotype (iNOS), present in activated macrophages and neutrophils (16). The iNOS is inducible by IFN-γ, TNF-α, Interleukin-1 (IL-1), and LPS (17). Unlike the constitutive form, the inducible isotype binds calmodulin with high avidity and hence it is essentially independent on added calmodulin for its action (18). Calmodulin copurifies with the iNOS following boiling in SDS, thus calmodulin acts as a constitutive subunit of the iNOS (18). Both isotypes convert L-arginine into L-citrulline and nitric oxide, and both are inhibited by many L-arginine analogues such as N-monomethyl arginine (L-NMMA), and aminoguanidine (AG) (19,20). The latter is very much more inhibitory to the iNOS rather than to the cNOS (21-23).

Abbreviations used in this paper: AG, aminoguanidine; BCG, Bacille Calmette-Guérin; cNOS, constitutive nitric oxide synthase; DAB, diaminobenzidine; iNOS, inducible nitric oxide synthase; NADPH, nicotinamide dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase.

Two signals are needed for an effective synthesis of nitric oxide by macrophages: a priming signal which is usually mediated by IFN-γ, provided by activated T-cells and/or Natural Killer cells, and a triggering signal that is delivered by either TNF-α or LPS (17). The rationale behind these studies proposes that decidual infiltration by activated NK cells and macrophages provides the necessary components for the priming of decidual macrophages and release of macrophage derived cytolytic factors. This study shows that decidual macrophages are primed *in vivo* during early pregnancy and that activated NO production is associated with early embryo loss.

Materials and Methods

Experimental Animals

DBA/2 males and CD1 mice were purchased from Charles River (St. Constant, Quebec) and CBA/J females were purchased from Jackson laboratories (Bar Harbour, Maine). The housing and handling of the experimental animals was in accordance with the guidelines of the Canadian Council for Animal Care. Four female mice were housed with a single male, and checked daily for the presence of a copulatory plug. The day of appearance of a mating plug was arbitrarily designated as day 0. Mice were then killed by cervical dislocation at days 8, 10, and 12 of pregnancy. To increase the basal incidence rate of resorption, a separate group of CBA/J females were injected intraperitoneally (i.p.) with 30 μg of poly I:C at day 6 of gestation. Treatment with poly I:C has been shown to increase fetal resorption up to 60% (2).

Primed macrophages were obtained from the spleen of non-pregnant mice 5 days after they were injected with $1.4X10^8$ viable Bacillus Calmette-Guerin (BCG).

Preparation of Decidual cell Suspension

The uteri were surgically removed and the number of viable implantation sites together with the number of resorbing embryos were counted. For days 10 and 12 embryos, the uterine tissues were peeled off and the implantation sites were separated from the embryos. At day 8, both the embryo and the implantation sites were used as sources of cells due to difficulties in separating them. Individual implantation sites were minced, in 1 ml of cold Hanks Balanced Salt Solution (HBSS), into very small pieces using a sterile Richard utility blade. The cell suspensions together with the tissue debris were filtered through a 93 μm Nylon mesh (Thompson B & SH, Montreal, Quebec). The filtrates were centrifuged at 200g for 10 minutes in a Sorval centrifuge. Contaminating red blood cells were disrupted in sterile lysing buffer (0.15M NH_4Cl) for 3 minutes at room temperature . The cells were then washed in HBSS. The last wash was done in nitrite, nitrate, and phenol red free Minimum Essential Medium (MEM) (Sigma, St. Louis, MO), supplemented with L-glutamate (2mM), penicillin (100U/ml), streptomycin (100 μg/ml) and 5% Fetal Calf Serum (ICN, Montreal, Quebec). The pellet was then resuspended at 1 $X10^6$/ml in nitrate free MEM. Generally, more than 90% of the cells were viable as shown by trypan blue exclusion, and about 10% stained with Mac-1 and F4/80 antibodies (data not shown). The cell suspension was then aliquoted to a 96 well flat-bottomed culture plate (100 μl/well) (Flow Laboratories, Virginia) and the cells were incubated with either 50 μl of meduim containing 10 μg of LPS (055:B5 E.coli, Sigma) or with meduim alone. In some of the experiments 1 mM of aminoguanidine (Sigma, St. Louis, MO) was added in a volume of 50 μl of meduim. After overnight incubation at 37°C in a 5% CO_2 incubator, the supernatants were harvested and tested for the presence of nitrite and nitrate.

Single and Double Immunohistochemistry

In this experiment frozen sections (8 μm thick) were prepared from day 8 uteri, were air dried and fixed in 2% Paraformaldehyde (Fisher Scientific, New Jersey). To reduce non-specific staining, sections were incubated with normal goat serum (Vector Laboratories, Burlingame) for 20 minutes at room temperature. In single-labelled immunostaining, washed sections were then incubated with rabbit anti-murine iNOS antibody (1:1000) (Transduction Laboratories, Kentucky) overnight at 4 °C. Sections were equilibrated at room temperature for 1 hour and then incubated with biotinylated anti-rabbit IgG antibody and later with avidin:biotinylated horseradish peroxidase complex (ABC) used according to the manufacture's instructions (Vector Laboratories, Burlingame). The colour was developed for 5 minutes with peroxidase substrate, diaminobenzidine (DAB) (Sigma), and counterstained with Mayer's hematoxylin (Sigma). Sections were then mounted with crystal/Mount (Biomedia, Foster city).

In double-labeled immunostaining, slides were simultaneously incubated with rat anti-Mac-1 antibody (American Type Culture Collection, Rockville) and rabbit anti-iNOS antibody. Washed sections were incubated with alkaline phosphatase conjugated goat anti-rat IgG (Sigma) together with horseradish peroxidase conjugated goat anti-rabbit IgG (Sigma). The sections were first stained with alkaline phosphatase substrate, 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium (BCIP/NBT) (Sigma). Levamisole (Sigma) was added to the substrate buffer to reduce the activity of the endogenous placental phosphatase. The washed sections were then incubated with DAB, a peroxidase substrate. Sections were then mounted with Crystal Mount (Biomedia, Foster City). In both staining methods endogenous peroxidase was quenched with a 15-minute incubation in 3% hydrogen peroxide solution (Sigma) in 100% methanol (American Chemicals, Montreal) following incubation with the primary antibody. As a negative control, sections were incubated with the second antibodies and substrate without exposure to primary antibody, or with the addition of a primary isotype control antibody.

Preparation of spleen cell suspension

Spleen cells from mice injected 5 days previously with BCG, were prepared by mincing the spleen in 5 ml of RPMI 1640 medium (GIBCO) supplemented with 5% fetal calf serum, 2mM L-glutamate, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cell suspensions were sedimented for 10 minutes on ice. The tissue debris was discarded and the cell suspension was centrifuged for 5 minutes at 200 g. The resulting pellet was then washed 3 times with HBSS. The cells were resuspended in RPMI at a concentration of 1 $X10^7$/ml and aliquots of 100 μl were placed in each well of a 96 well flat-bottomed plate in the presence or absence of 10 μg of LPS at 37°C and 5% CO_2.

Assay for nitric Oxide

Nitric oxide produced by activated macrophages accumulates as nitrite (NO_2^-) and nitrate (NO_3^-) in the culture supernatant. Nitrite in 50 μl of the culture supernatants were assayed with Greiss reagent (24). Briefly, equal amounts (50 μl) of the supernatant and Greiss reagent (Sulphonamide, 1% in 2.5% H_3PO_4, and (1-Naphthyl)Ethylenediamine, 0.1% in water) were allowed to react in flat-bottomed

96 well culture plates, with gentle mixing, for 10 minutes at room temperature. The colored product was read on a Titertek Multiskan reader at 540nm using as 670nm readings at reference wavelength to compensate for non-specific absorbance. The concentration of nitrite in the samples were determined as μM of NO using the standard curve for nitrite included in each assay. Where total NO production as both nitrite and nitrate were assayed, nitrate was first reduced to nitrite and then detected by the Greiss reagent using a kit obtained from Boehringer Mannheim. Briefly, the reduction of nitrate to nitrite was achieved by using nitrate reductase enzyme purified from Aspergillus species in the presence of NADPH. Twenty microliters of a NADPH solution, prepared by dissolving 0.5 mg of NADPH in 400 μl ddH_20, was added to 50 μl of the supernatant followed by 9 μl of purified nitrate reductase (5U/ml). The mixture was incubated for 20 minutes at room temperature and the reduced nitrate was then detected by the Greiss reagent as described above. The concentration of nitrate was deduced from a nitrate standard curve. In effect, this assay measured total NO production.

Treatment of mice with aminoguanidine

Pregnant CBA/J females mated by DBA/2 males were injected at day 6 with 30 μg of poly I: C. From day 6 to day 10, the mice were injected twice daily with 6 mg of aminoguanidine (AG) (i/p), while control mice received saline. Alternatively, to avoid the trauma associated with injecting mice, AG was added to the drinking water (0.6 g/ 100 ml) from day 6 to day 10. The mice were killed at day 12, and the number of implantation sites together with the number of resorbing embryos were counted as described above. The proportion of resorbing embryos was presented as a percentage as derived from the formula: 100 X Resorbing embryos/ (Viable plus resorbing embryos). A separate group of mice were allowed to survive to term and the number of delivered embryos were enumerated and examined for gross morphologic defects.

Results

Production of Nitric Oxide is a Sensitive Indicator of Macrophage Priming

To demonstrate the utility of NO as an indicator of the priming of macrophage *in vivo*, splenocytes were isolated from BCG primed mice and challenged with LPS *in vitro*. It was apparent that the augmented ability of macrophages to produce NO was a sensitive measure of macrophage activation. Resting macrophages produced little NO whether or not they were challenged with LPS *in vitro*, while BCG primed splenic macrophages produced significant amounts of NO when challenged with LPS compared to the BCG primed macrophages alone (fig 1). Furthermore, the quantity of nitrite produced was directly proportional to the number of spleen cells incubated (Data not shown). These results showed that both a priming signal and a triggering signal were needed for macrophages to produce nitric oxide. Therefore, the production of NO by mononuclear cells in response to *in vitro* challenge with LPS could be used as a sensitive indicator of the priming of macrophages.

Nitric Oxide is Produced by Decidual Cells from Resorbing Embryos

In this study, we investigated the role of NO as an effector molecule in the mechanism of early fetal rejection. Assays of NO production by cells at the implantation sites from CBA/J female mice indicated that decidual mononuclear cells were

primed *in vivo* and were responsive to challenge by LPS (fig 2). Furthermore, decidual cells from resorbing embryos at day 12 of gestation produced significantly more NO than non-resorbing embryos. Likewise, decidual cells from CD1 mice produced similar concentrations of nitric oxide, and moreover, resorbing embryos from CD1 female mice showed elevated quantities of NO (fig 2). Therefore, production of NO appears to be a good marker for embryo resorption.

The results documented in figure 2 showed that implantation sites taken from day 12 resorbing embryos of both CBA/J (fig 2a) and CD1 (fig 2b) produced significantly more NO as compared to non-resorbing embryos. When the data was plotted as a frequency histogram, the production of nitrite by individual embryos from both CBA/J and CD1 females at day 12 of gestation showed a distinct bimodal distribution (fig 3). This bimodal distribution was also seen when day 12 CBA/J embryos were tested for the production of nitrate (fig 4). However, it was clear from this data that nitrate was a more sensitive indicator of total nitric oxide production than nitrite alone, and for subsequent assays nitrate was measured.

All the resorbing embryos from pregnant CBA/J mice produced between 30 and 70 μM of nitrate per 10^5 cells decidual mononuclear cells. All the non-resorbing embryos produced between 0 and 30 μM nitrate. Therefore, the nitrate production by the non-resorbing embryos was used to define the limits of normal nitrate production for the analysis of embryos at earlier stages of development. The day 12 non-resorbing embryos produced an average of 11.23 ± 6.12 μM of nitrate and the upper 95% confidence limit for nitrate production was computed as 22 μM/10^5 decidual cells. Using this value, nitrate production by individual embryos at day 8 and day 10 of gestation were categorized as being normal or significantly increased. Table 1 shows data for CBA/J X DBA/2 matings in which an average of 23 % of embryos between day 8 and 12 showed increased nitrate production. Similarly, 3 % of embryos from CD1 X CD1

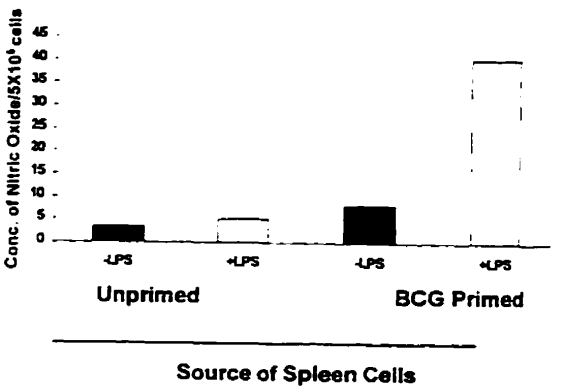

Figure 1. Nitric oxide production by BCG-primed spleen cells. Mice were injected intraperitoneally with 1.4×10^6 BCG organisms or PBS, and 5 d later, spleen cells were collected (1×10^7/ml) and challenged with LPS in vitro. Culture supernatants from 1×10^6 spleen cells per well incubated with 10 μg of LPS per well were harvested and assayed for NO accumulation as nitrite using the Greiss reagent. *Open bars*, nitric oxide production by cells challenged with LPS; *closed bars*, cells challenged with medium alone. Results expressed as μM NO/5×10^5 cells.

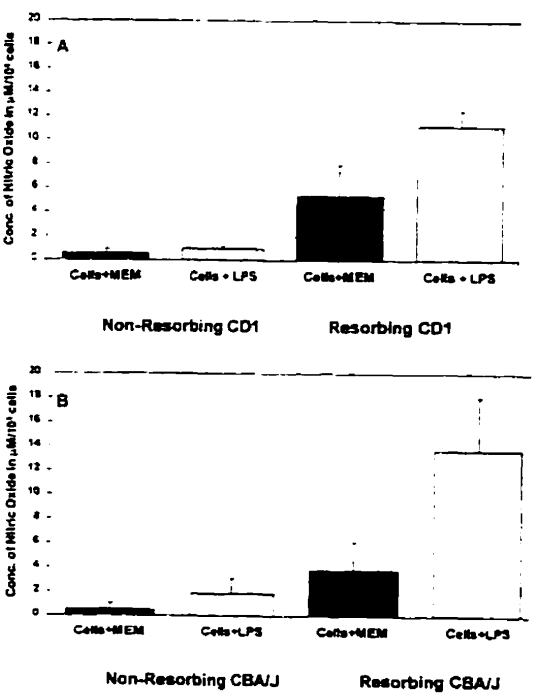

Figure 2. Nitric oxide production by cells from implantation sites taken from CBA/J (*A*) and CD1 (*B*) resorbing and nonresorbing embryos on day 12 of gestation. Pregnant female mice were killed on day 12 of gestation, and cells (10^5 in 100 μL) from individual implantation sites of resorbing and nonresorbing embryos were prepared for in vitro challenge with LPS. Culture supernatants were assayed for the presence of NO as nitrite, and the results were expressed as micromoles of NO in the culture supernatant. *Open bars*, NO produced by LPS-challenged cell cultures; *closed bars*, cells cultured with PBS. Results are presented as means ± SD for assays performed using 4 CBA/J and 11 CD1 mice.

matings showed elevated levels of nitrate production between day 8 and 12. Therefore, in both of mating combinations the production of significant concentration of nitrate was associated with embryo loss. More interestingly, decidual cells from CBA/J X DBA/2 embryos at day 8 and day 10 produced significant amounts of nitrate even in the absence of LPS suggesting that NO produced damage may have already begun as early as day 8 in these embryos. Therefore, increased NO production by cells at the implantation sites preceded visible embryo resorption.

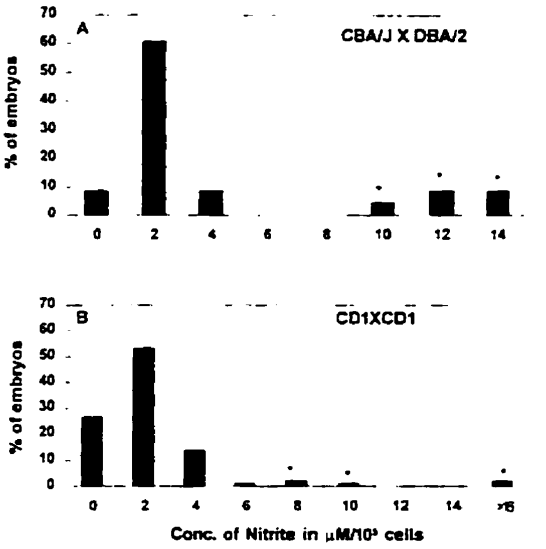

Figure 3. The frequency of distribution of day 12 embryos versus nitrite production. Cells from implantation sites from resorbing and nonresorbing embryos were individually tested for NO production as nitrite on day 12 in 4 CBA/J females mated with DBA/2 males (*A*), and 11 CD1 females mated with CD1 males (*B*). The results are expressed as micromoles NO in the culture supernatant. Bars indicate the number of embryos producing between 0 and 16 µM of nitrite by 10^5 decidual cells by 2-µM intervals. *Groups of day 12 embryos that were all resorbing.

Expression of iNOS Protein by Decidual Mononuclear Cells

To investigate the presence of iNOS in pregnant mouse uteri, implantation sites from CBA/J females mated with DBA/2 males mice were stained with rabbit anti-mouse iNOS antibody. Fig (5a) shows a representative view of a day 8 embryo, where positive cells were observed in the

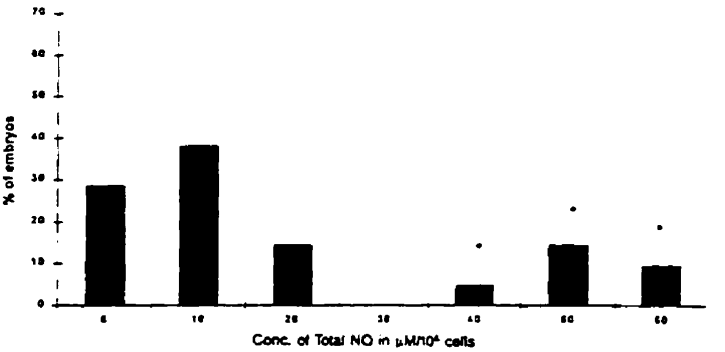

Figure 4. The frequency distribution of day 12 CBA/J embryos vs total nitric oxide production (nitrite plus nitrate). Cells from implantation sites from resorbing and nonresorbing embryos taken from four CBA/J females were individually tested for total NO production as both nitrite and nitrate. The results are expressed as micromoles of NO in the culture supernatant. Bars indicate the number of embryos producing between 0 and 70 µM of NO by 10^5 decidual cells by 10-µM intervals. *Groups of day 12 embryos that were all resorbing.

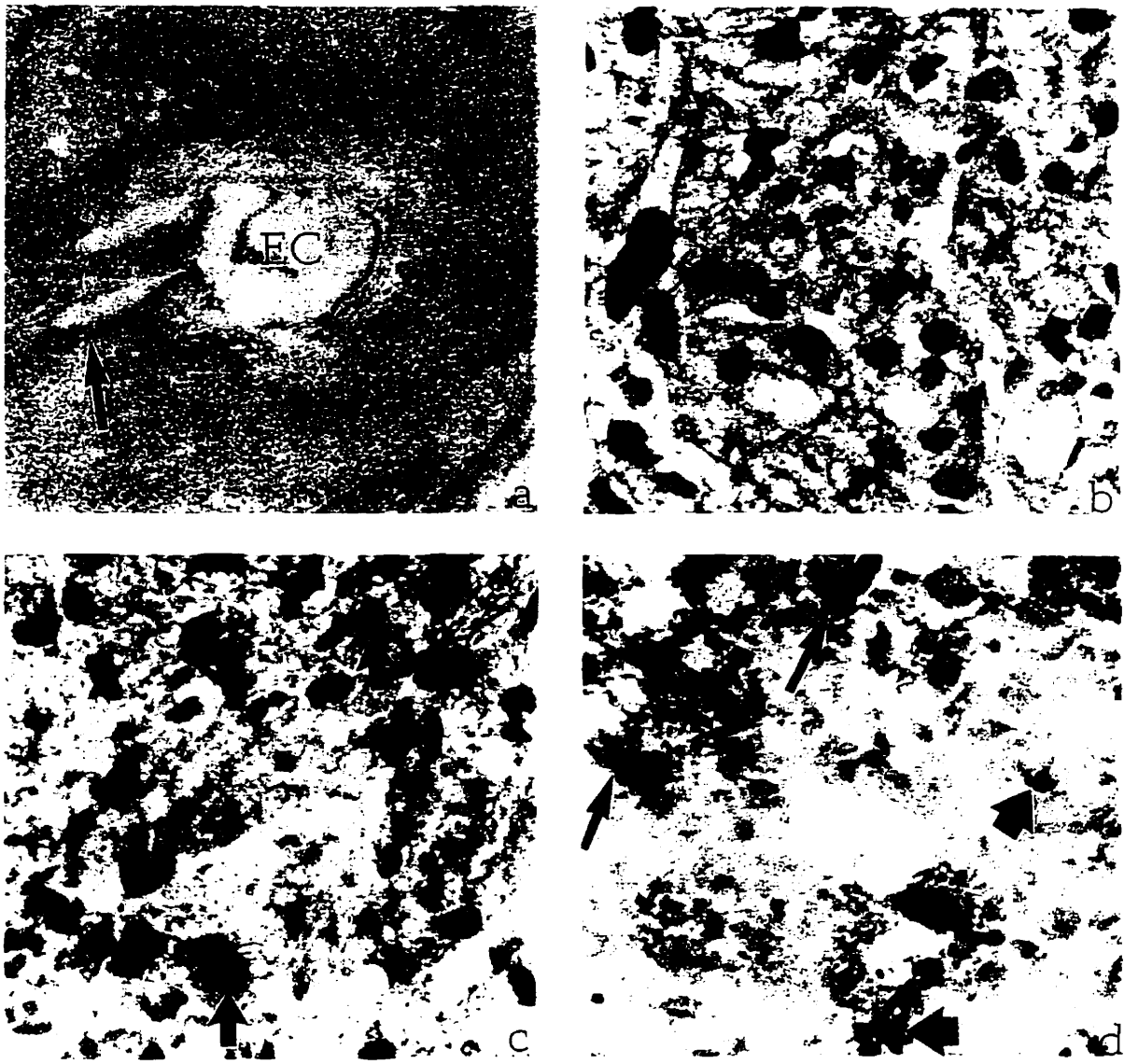

Figure 5. Detection of iNOS in mouse decidua. Frozen sections were air dried for 6 h, fixed in 2% paraformaldehyde for 30 min, and washed with Tris-HCl, pH 7.46, before staining. Sections were treated with (*a* and *c*) and without (*b*) anti-iNOS antibody, respectively, stained with DAB, and counterstained with Mayer's hematoxylin. (*a*) A low power view of iNOS staining in a day 8 embryo (×3.2). The embryonic capsule (*EC*) and the area of positive staining (*arrow*) are indicated. (*b*) A negative control stain in which the primary antibody was not added. (*c*) A population of iNOS-positive cells (*arrows*) surrounding the embryonic capsule (not shown) at ×600. (*d*) Sections were treated with both rabbit anti-iNOS and rat anti-Mac-1 antibodies at the same time. Horseradish peroxidase–conjugated goat anti–rabbit and alkaline phosphatase–conjugated goat anti–rat were then added. DAB (*brown*) and BCIP/NBT (*blue*) were substrates for the peroxidase and the phosphatase, respectively. Cells with single staining for Mac-1 (*large arrows*) and double staining (*thin arrows*) for Mac-1 and iNOS are indicated (×600).

decidua. At a magnification of 600, a population of iNOS-containing cells was seen around the embryonic capsule (Fig. 5 *c*). A negative control section with no primary antibody showed no significant staining (Fig. 5 *b*). To determine the cellular source of NO production by cells at the implantation sites, sections were stained with rabbit anti–mouse iNOS together with rat anti–Mac-1 antibodies. Fig. 5 *d* clearly shows a distinct population of double-stained cells. Some cells that stained only for Mac-1 were also seen in the same area.

Inhibition of NO Production Reduces Embryo Losses. To confirm the involvement of NO in early embryo loss, AG was used to inhibit the production of NO in vivo. AG was either injected or given orally in the drinking water to

Table 1. *Production of Nitrate by Embryos from CBA/J × DBA/2 Matings between Days 8–12 of Gestation*

	CBA/J mice tested	Implantation site per mouse	Number of embryos that produced nitrate without LPS*	Number of embryos that produced nitrate with LPS*	Percent of embryos with significant nitrate production with LPS
Day 8	5	6.2 ± 1.3	3	9	29.0%
Day 10	4	8.5 ± 2.0	1	6	17.6%
Day 12	4	6.3 ± 1.3	1	5	20.0%

*Nitrate production above the upper 95% confidence limit (UCL 95%) established for day 12 nonresorbing embryos (>22 μM/10^5 cells) was considered significant.
Mice were killed on day 8, 10, or 12 of gestation. Embryos were minced in nitrite- and nitrate-free medium, and the cell suspensions were challenged with LPS in vitro for 24 h. Reduction of nitrate to nitrite was achieved using nitrate reductase in the presence of NADPH.

gravid CBA/J female mice starting at day 6 of gestation. In both cases, aminoguanidine significantly reduced the resorption rate of CBA/J females mated with DBA/2 males (table 3). This was determined by counting the number of dead embryos at day 12 of gestation. To show that AG increased embryo survival and litter size, aminoguanidine treated mice were allowed to go to term. Table 4 shows a significant in average litter size of AG treated mice as compared to untreated controls. These results suggested that the production of NO by cells at the implantation sites was a major mediator of early embryo death.

Discussion

In this study, nitrogen oxide, a macrophage effector molecule, was shown to be involved in the mechanism associated with early embryo loss. Cells at the implantation sites from CBA/J and CD1 female mice were tested for nitrite and nitrate production by the Greiss reagent. At day 12 of gestation, resorbing embryos from both strains showed more than a 5 fold increase in LPS induced NO production compared to non-resorbing embryos. On the other hand, resorbing embryos were also shown to produce significant basal amounts of NO in the absence of LPS. This was not surprising, since at day 12 resorbing embryos were already damaged and effector cells were assumed to be primed and activated. To demonstrate the involvement of NO in the mechanism of early embryo loss prior to pathologic changes in the embryo, we tested the production of nitrite and nitrate by placental cells at earlier days of gestation.

Table 2. *Production of Nitrate by Embryos from CD1 × CD1 Matings between Days 8–12 of Gestation*

	CD1 mice tested	Implantation site per mouse	Number of embryos that produced nitrate without LPS*	Number of embryos that produced nitrate with LPS*	Percent of embryos with significant nitrate production with LPS
Day 8	4	11.3 ± 2.2	1	2	4.4%
Day 10	4	12.8 ± 1.7	0	0	0.0%
Day 12	11	10.7 ± 3.3	2	5	4.3%

Cell from implantation sites of CD1 mice were treated in the same manner as those from CBA/J females as described in the legend for Table 1.
*Nitrate production above the upper 95% confidence limit (UCL 95%) established for day 12 nonresorbing embryos (>22 μM per 10^5 cells) was considered significant.

Table 3. *AG-induced Reduction of Early Embryo Loss in CBA/J Female × DBA/2 Male Matings*

	Number of mice	Implantation sites per mouse	Viable embryos per mouse	Resorption rate (%)
Control mice PBS injection (i.p.)	5	8.0 ± 0.7	5.8 ± 1.0	27.1 ± 7.3
AG by injection (i.p.)	5	8.8 ± 2.4	8.0 ± 2.2	9.1 ± 6.0*
AG in drinking water	4	9.3 ± 1.5	8.5 ± 1.5	10.0 ± 5.7*

*$p < 0.05$ compared to control. Using Student's t test, treated mice showed a significant decrease in resorption rates. CBA/J pregnant females were injected with 50 μg of polyI:C on day 0. From 6 until day 10, the mice were injected twice daily with 6 mg AG. Control mice received saline. A separate group of mice received AG (40 mM) in drinking water. The number of implantation sites together with the number of resorbing embryos were counted on day 12 of gestation.

Table 4. *AG-treated CBA/J × DBA/2 Matings Showed Increased Litter Size*

	Number of mice	Litter size
Control mice	10	6.3 ± 1.42*
AG-treated mice	10	8.1 ± 1.45*

*$P < 0.05$ compared to control using Student's t test. AG (40 mM) was provided in drinking water. AG-treated mice showed a significant increase in average litter size compared to control (mean ± SD). The litter were allowed to come to term, killed between 4 and 28 d after birth, and examined for gross morphological defects. No obvious morphological defects were seen in either group.

Our results showed that the percentage of implantation sites from day 8 or day 10 pregnant CBA/J and CD1 mice that were capable of producing NO corresponded to the natural incidence of resorption observed in both strains. Of note, we often detected considerably more nitrate than nitrite in these embryos. Similar preferential production of nitrate has been reported in mice infected with malaria (25). This may have been due to the high oxidative capacity of the activated cells, that may have rapidly oxidized nitrite to nitrate. Furthermore, an increase in nitrite oxidation to nitrate by decidual cells could be one way of protecting embryos from the harmful effects of NO/NO_2^-. More interestingly, we detected an increased production of NO in the absence of LPS at days 8 and 10. This suggested that by day 8, the effector cells were already activated and the damage may already have started.

The cellular source of NO production and hence the specific effector cells have not been fully explored in pregnant mice. Nitric oxide can be constitutively produced by many cells including vascular endothelial cells in placental tissues, and this may argue against an inducible NOS as the source of the observed NO production. However, our results showed that a more than 5 fold increase in NO production by cells at implantation sites was induced by LPS implicating the activation of the inducible NOS as a likely source of the NO produced. Furthermore, the placental cells from the resorbing embryos produced significantly more NO when challenged by LPS *in vitro,* further suggesting activated iNOS as the effector mechanism leading to embryo demise. Conversely, we were able to detect basal (constitutive) nitrite and nitrate production by non-resorbing placentas at day 12, and trace amounts at day 10 of gestation. This constitutive NO production may have been due to the normal role that NO plays in uterine muscle relaxation (26). On the other hand, neutrophils can be induced to produce NO (27), however their involvement in early embryo loss is unlikely since these cells were not notably present at days 8 and 10 of gestation (28). Nevertheless, using immunohistochemistry, we were able to detect a significant increase in F4/80 positive cells, a selective marker for macrophages, in resorbing compared to non-resorbing embryo (4). Furthermore, approximately 10% of the dispersed decidual cells expressed the Mac-1 and F4/80 markers.

To further investigate the nature of the nitric oxide synthase involved in early embryo loss, we tested the presence of iNOS in frozen tissue sections of pregnant uteri by immunohistochemistry. These studies confirmed our observation of the involvement of a macrophage associated inducible form of NOS in early embryo loss. In addition, uterine sections were also double stained for both iNOS and a macrophage marker, Mac-1 (CD11b). As expected, iNOS positive cells also stained with Mac-1, indicating decidual mononuclear cells as a likely cellular source of NO production. Some single stained Mac-1 positive cells (iNOS negative) were also detected in day 8 decidua. Since few T-cells or neutrophils have been observed in day 8 implantation sites, these cells may have been inactivated macrophages or natural killer cells (4). For these reasons, it appeared that in this study, a macrophage associated iNOS was involved in the production of nitric oxide at the implantation sites.

To demonstrate the involvement of NO production in early embryo loss, the NOS inhibitor aminoguanidine (AG), was used to inhibit NO production *in vivo*. Aminoguanidine was shown to be a selective inhibitor for the iNOS which was mainly present in activated macrophages, as opposed to the cNOS present in vascular endothelial cells and neurons (21-23). NO production was virtually completely abrogated when placental cells were incubated with AG *in vitro* (data not shown). More striking results were observed when mice were given AG *in vivo*. The resorption rate in CBA/J females was reduced from 28% to 11% when mice were either injected with AG or given AG in drinking water. These results directly showed the involvement of NO in the effector mechanisms of fetal rejection. When aminoguanidine treated mice were allowed to proceed to term, there was a significant increase in average litter size as compared to non-treated mice. The litters appeared morphologically normal when examined between birth and 4 weeks of age. These studies showed that embryos which survive to day 12 of gestation, usually survive to term without any significant additional losses since the number of viable embryos/mouse at day 12 and at delivery were not statistically different. The mechanisms of early fetal rejection are regulated by a complex network of cytokines. It is known that only primed and not resting macrophages produce NO when challenged with either LPS or TNF-α (29). Interferon-γ primed macrophages *in vivo* as well as *in vitro* (30). Our data showed that placental mononulear cells in potentially resorbing embryos were primed and could produce NO when challenged with LPS *in vitro*. The source of the priming molecule could be IFN-γ, or TNF-α which may be produced by infiltrating NK cells. In fact, injecting gravid mice with IFN-γ or Poly I:C, an activator of IFN production, was shown to increase the resorption rate in CBA/J females when mated with DBA/2 males (2). Poly I:C could therefore have indirectly increased decidual macrophage priming by inducing IFN-γ and/or TNF-α production by decidual NK cells, and hence an increase in NO production. Furthermore, the association of NK cells with early embryo loss has been observed (5). Tumor necrosis factor-α, which is also produced by activated macrophages, was associated with embryo resorption in CBA/J X DBA/2 mice (6,10). We have previously reported the production and the release of TNF-α within the pregnant uterus and that embryo loss was reduced by the TNF-α inhibitor, pentoxifylline (6). Similarly, others showed that inoculation of gravid mice with recombinent TNF-α induced early embryo loss (10). The presence at the implantation sites of both IFN-γ and TNF-α, which cooperatively promote nitric oxide production by macrophages, implies that a macrophage inducible nitric oxide synthase is involved in early embryo loss.

The results in this work demonstrate the involvement of NO in early embryo loss and implicates the activation of primed decidual macrophages in effecting spontaneous abortion.

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References

1. Clark, D.A., M.R. McDermott, and M.R. Szewczuk. 1980. Impairment of host-versus-graft reaction in pregnant mice. II. Selective suppression of cytotoxic T-cell generation correlates with soluble suppressor activity and with successful allogeneic pregnancy. *Cell. Immunol.* 52:106–118.
2. de Fougerolles, A.R., and M.G. Baines. 1987. Modulation of the natural killer cell activity in pregnant mice alters the spontaneous abortion rate. *J. Reprod. Immunol.* 11:147–153.
3. Duclos, A.J., D.K. Pomerantz, and M.G. Baines. 1994. Relationship between decidual leukocyte infiltration and spontaneous abortion in a murine model of early fetal resorption. *Cell. Immunol.* 159:184–193.
4. Duclos, A.J., E.K. Haddad, and M.G. Baines. 1994. Presence of activated macrophages in the decidua of early embryos prior to spontaneous resorption. *Am. J. Reprod. Immunol.* 33: 354–366.
5. Gendron, R.L., and M.G. Baines. 1988. Infiltrating decidual natural killer cells are associated with spontaneous abortion in mice. *Cell. Immunol.* 113:261–267.
6. Gendron, R.L., F.P. Nestel, W.S. Lapp, and M.G. Baines. 1990. Lipopolysaccharide-induced fetal resorption in mice is associated with the intrauterine production of tumour necrosis factor-alpha. *J. Reprod. Fertil.* 90:395–402.
7. Hunt, J.S., L.S. Manning, M.D. Mitchell, J.R. Selanders, and G.W. Wood. 1985. Localization and characterization of macrophages in murine uterus. *J. Leukocyte Biol.* 38:255–261.
8. Bulmer, J.N., D.P. Lunny, and S.V. Hagin. 1988. Immunohistochemical characterization of stromal leucocytes in non-pregnant human endometrium. *Am. J. Reprod. Immunol. Microbiol.* 17:83–90.
9. Hunt, J.S. 1990. Current topics: the role of macrophages in the uterine response to pregnancy. [Review]. *Placenta.* 11: 467–475.
10. Chaouat, G., E. Menu, D.A. Clark, M. Dy, M. Minkowski, and T.G. Wegmann. 1990. Control of fetal survival in CBA × DBA/2 mice by lymphokine therapy. *J. Reprod. Fertil.* 89: 447–458.
11. Naume, B., A.C. Johnsen, T. Espevik, and A. Sundan. 1993. Gene expression and secretion of cytokines and cytokine receptors from highly purified CD56$^-$ natural killer cells stimulated with interleukin-2, interleukin-7 and interleukin-12. *Eur. J. Immunol.* 23:1831–1838.
12. Beutler, B., N. Kronchin, I. Milsark, C. Luedke, and A. Cerami. 1986. Control of cachectin (tumor necrosis factor) synthesis: mechanisms of endotoxin resistance. *Science (Wash. DC).* 232:977–980.
13. Burchett, S.K., W.M. Weaver, J.A. Westall, A. Larsen, S. Kronheim, and C.B. Wilson. 1988. Regulation of tumor necrosis factor/cachectin and IL-1 secretion in human mononuclear phagocytes. *J. Immunol.* 140:3473–3481.
14. Geller, D.A., A.K. Nussler, M. Di Silvio, C.J. Lowenstein, R.A. Shapiro, S.C. Wang, R.L. Simmons, and T.R. Billiar. 1993. Cytokines, endotoxin, and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proc. Natl. Acad. Sci. USA.* 90:522–526.
15. Moncada, S., R.M.J. Palmer, and E.A. Higgs. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharm. Rev.* 43:109–141.
16. Knowles, R.G., and S. Moncada. 1994. Nitric oxide synthases in mammals. [Review]. *Biochem. J.* 298:249–258.
17. Ding, A.H., C.F. Nathan, and D.J. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol.* 141:2407–2412.
18. Cho, H.J., Q.W. Xie, J. Calaycay, R.A. Mumford, K.M. Swiderek, T.D. Lee, and C. Nathan. 1992. Calmodulin is a subunit of nitric oxide synthase from macrophages. *J. Exp. Med.* 176:599–604.
19. Nathan, C.F., and Q.W. Xie. 1994. Nitric oxide synthases: roles, tolls, and controls. *Cell.* 78:915–918.
20. Moncada, S., and A. Higgs. 1993. The L-arginine-nitric oxide pathway. [Review]. *N. Eng. J. Med.* 329:2002–2012.
21. Griffiths, M.J., M. Messent, R.J. MacAllister, and T.W. Evans. 1993. Aminoguanidine selectively inhibits inducible nitric oxide synthase. *Br. J. Pharmacol.* 110:963–968.
22. Hasan, K., B.J. Heesen, J.A. Corbett, M.L. McDaniel, K. Chang, W. Allison, B.H. Wolffenbuttel, J.R. Williamson, and R.G. Tilton. 1993. Inhibition of nitric oxide formation by guanidines. *Eur. J. Pharmacol.* 249:101–106.
23. Joly, G.A., M. Ayres, F. Chelly, and R.G. Kilbourn. 1994. Effects of NG-methyl-L-arginine, NG-nitro-L-arginine, and aminoguanidine on constitutive and inducible nitric oxide synthase in rat aorta. *Biochem. Biophys. Res. Commun.* 199: 147–154.

24. Green, L.C., D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok, and S.R. Tannenbaum. 1982. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal Biochem.* 126:131–138.
25. Rockett, K.A., M.M. Awburn, B.B. Aggarwal, W.B. Cowden, and I.A. Clark. 1992. In vivo induction of nitrite and nitrate by tumor necrosis factor lymphotoxin and interleukin-1: possible roles in malaria. *Infect. Immun.* 60:3725–3730.
26. Conrad, K.P., M. Vill, P.G. McGuire, W.G. Dail, and A.K. Davis. 1993. Expression of nitric oxide synthase by syncytiotrophoblast in human placental villi. *Fed. Am. Soc. Exp. Biol. J.* 7:1269–1276.
27. McCall, T.B., N.K. Boughton-Smith, R.M. Palmer, B.J. Whittle, and S. Moncada. 1989. Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. *Biochem. J.* 261:293–296.
28. De, M., R. Choudhuri, and G.W. Wood. 1991. Determination of the number and distribution of macrophages, lymphocytes, and granulocytes in the mouse uterus from mating through implantation. *J. Leukocyte Biol.* 50:252–262.
29. Stuehr, D.J., and M.A. Marletta. 1987. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-gamma. *J. Immunol.* 139:518–525.
30. Dalton, D.K., S. Pitts-Meek, S. Keshav, I.S. Figari, A. Bradley, and T.A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science (Wash. DC).* 259:1739–1742.

Preface to chapter 3

We have previously shown that decidual activated macrophages were responsible for the production of nitric oxide and embryo loss. Two signals are needed for the production of nitric oxide by macrophages, where IFN-γ has been shown to serve as the first signal. In this study, we investigated the role of IFN-γ in early embryo loss as a potential first signal needed for decidual macrophage activation and subsequent embryo death.

Interferon-γ Primes Macrophages for Nitric Oxide production and Fetal Abortion: In Vivo Role of IFN-γ in Early Embryo Loss[1]

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Abstract

We have previously shown that both priming and triggering signals were needed for nitric oxide production by activated decidual macrophages and that nitric oxide production was associated with embryo death. In this study, we investigated the role of IFN–γ as the primary signal for macrophage activation in early embryo loss. Interferon-γ-deficient (GKO) and heterozygous F1 control mice were injected with lipopolysaccharide (LPS) at day 7 of gestation. The results showed that 12% of the embryos from the IFN-γ deficient and 47% of the embryos from the control mice resorbed when mice were examined at day 12 of gestation. This suggested that IFN-γ was needed for LPS induced embryo resorption and that decidual macrophages from pregnant GKO mice were not primed and could not be activated when given LPS. Further, the results showed that IFN-γ mRNA was simultaneously expressed in the same embryos that also expressed mRNA markers for macrophage priming (TNF-α and iNOS) indicating that macrophage activation and subsequent nitric oxide production could be consequence of IFN-γ production. Similarly, we investigated the role of IL-12 as a switch cytokine capable of eliciting TH1 associated cytokine production including IFN–γ. The results showed that IL-12 mRNA expression was observed in embryos that simultaneously showed IFN–γ, TNF–α, and iNOS expression, indicating that induction of TH1

cytokine production and macrophage activation could have been the result of IL-12 expression. In this *in vivo* study, we showed for the first time that decidual IFN-γ expression is detrimental to embryo survival. The results also suggested that IFN-γ could provide the priming signal for decidual macrophages that lead to macrophage activation and consequent embryo death.

Introduction

In mammalian pregnancies, fetal survival may be influenced by the maternal immune system. In human, early embryo loss is defined as losses that occur before the third week of gestation and, therefore such losses are often unnoticed clinically. To study the early cellular and molecular events that lead to early embryo loss, many researchers have used the CBA/J females mated with DBA/2 males mouse mating model that spontaneously resorb 20%-30% of the embryos in each pregnancy (1,8,12). The resorption rate can be further increased 2-3 fold if the pregnant mice are treated with double stranded RNAs that induce interferon production and enhance natural killer (NK) cell activity (2,31). The mechanism of enhancement of early embryo loss by poly innosinic: cytidylic acid (poly I:C) is not fully understood, but shows a similar time course and cytopathology to that observed for spontaneous embryo loss in untreated CBA/J females mated with DBA/2 males (3,10). Using this model, we showed that early embryo loss was associated with the activation of non-specific innate mechanisms of maternal resistance (4).

We have previously shown that natural killer (NK) cells and macrophages were harmful to pregnancy. We reported that the number of activated macrophages were significantly increased in resorbing compared to healthy embryos (3,10). Similarly, we showed that NK cells

heavily infiltrated resorbing embryos prior to embryo loss (9). In addition, deleting NK cells by anti-NK cell antibody enhanced embryo survival during pregnancy (31). Recently, we have shown that nitric oxide (NO), produced by macrophage associated inducible nitric oxide synthase (iNOS), could directly mediate damage to a growing embryo (23). We further showed that decidual macrophages at the implantation sites were activated, as indicated by the expression of iNOS before embryo damage was apparent (23). More importantly, it has been shown that both a priming and a triggering signal were needed to induce NO production by activated macrophages (25). Many *in vivo* and *in vitro* experiments strongly suggested that IFN-γ was the principle priming signal for macrophages and that either LPS or TNF-α could be serve as the triggering signal (14,25).

Interferon-γ is a pleiotropic cytokine produced mainly by Th1 cells, and NK cells (17,19,21). Interferon-γ is the principle macrophage activating cytokine though other cytokines may also activate macrophages less efficiently (6). It has been observed that maternal cytokines play a critical role in determining the survival or death of a growing embryo (13). There is increasing evidence that Th1 cytokines such as IFN-γ and TNF-α are deleterious to pregnancy and Th2 cytokines including IL-4 and IL-10 are beneficial (27). As a protective measure, the maternal systemic immune response is generaliy influenced towards the

production of Th2 cytokines, such as IL-10, IL-3 and IL-5 (22). As a consequence, Th1 cytokine production including IFN-γ and TNF-α is significantly decreased (28). It is not yet clear what factor(s) mediate the switch to TH1 or TH2 cytokines. Some researchers have suggested that the nature of the antigen presented by the macrophages plays a fundamental role in determining the kind of cytokines to be produced by the T-cells (7,15). Evidence had also suggested that soluble factors such as IL-4 or IL-12 in the primary culture induced TH2 or TH1 cytokines production respectively (16).

Moreover, previous studies had shown that the inoculation of pregnant females with recombinant IFN-γ and/or TNF-α enhanced embryo resorption (29), whereas anti-TNF-α antibody and Pentoxifylline, known to inhibit TNF-α production and bioactivity, increased embryo survival (30). It should be noted that LPS, which could serve as the triggering signal for macrophage activation, also significantly augmented embryo resorption in most mouse strains when given intravenously to pregnant females (11,30).

In this report we demonstrated, for the first time, the *in vivo* role of IFN-γ in early embryo loss. The rationale behind these studies was to show that when depleting the primary signal (IFN-γ knockout mice or anti-IFN-γ antibodies), mice were resistant to LPS induced early embryo

loss. We also showed that the production of IFN-γ was associated with the presence of IL-12.

Materials and Methods

Animals and matings

Mice homozygous for the mutation in the IFN-γ genes (gko) were obtained from Genentech, Inc. (San Francisco, CA). The gko mice was expressed in Balb/c mice which were used as a breeding stock to generate both homozygous gko/gko and heterozygous (gko/-) wild type females. Tail DNA isolated from these mice were screened by PCR to detect IFN-γ genes using primers supplied by Genentech to confirm the identity of gko/gko and (gko/-) heterozygous mice. CBA/J females were purchased from The Jackson Laboratory (Bar Harbor, Maine) at an age of 8 weeks. DBA/2 males were acquired from Charles River (St. Constant, Quebec). The animals were allowed to adjust to the housing conditions for 6-8 weeks, and then mated. The housing and handling of the experimental animals was in accordance with the guidelines of the Canadian Council for Animal Care.

CBA/J, gko/gko, and (gko/-) females were mated with DBA/2 males and checked daily for the presence of a copulatory plug. The day

of appearance of a mating plug was arbitrarily designated as day 0. To determine the extent of priming of decidual macrophages, pregnant gko/gko and (gko/-) females received 10 μg of LPS intravenously at day 7 of gestation. In experiments to determine the requirement for IFN-γ, pregnant CBA/J females mated with DBA/2 males received intraperitoneal injection of either 100 μl of XMG1.2 (kindly provided by Dr. Trevor Owens) or isotype-matched control (Sigma) at day 6 of gestation. All mice were killed at day 12 of gestation and the resorbing and non-resorbing embryos were enumerated. Percentage of embryo loss was calculated from the formula: 100 X Resorbing embryos/ (Viable plus Resorbing embryos).

A separate group of gko/gko and (gko/-) pregnant females received intravenous (i.v.) injections of 100 μg of Poly I:C on day 7 to augment embryo losses (Sigma, St. Louis, MO). Treatment with poly I:C has been previously shown to increase the incidence of fetal resorption 2-3 fold (31).

To test for priming, gko/gko and (gko/-) mice received an intraperitoneal injection of 50 cysts containing the larval stage of *Echinococcus multiocularis*. Seven days later, peritoneal cells were collected and cultured *in vitro* with or without 5 μg/ml LPS and incubated for 18 hours at 37 °C in 5% CO_2 incubator and then assayed for NO production.

Nitric Oxide Assay

Nitric oxide produced by activated macrophages accumulates as nitrite (NO_2^-) and nitrate (NO_3^-) in the culture supernatant. Nitrite in the culture supernatants were assayed with the Greiss reagent (5). Briefly, equal amounts (50 µl) of the supernatant and Greiss reagent (Sulphonamide, 1% in 2.5% H_3PO_4, and (1-Naphthyl)Ethylenediamine, 0.1% in water) were allowed to react in flat-bottomed 96 well culture plates, with gentle mixing, for 10 minutes at room temperature. The colored product was quantified with a Titertek Multiskan reader at 540 nm using 670 nm as a reference wavelength to compensate for non-specific absorbance. The concentration of nitrite in the samples were determined as µM of NO/ml using a standard curve for nitrite which was included in each assay.

Primers and Probes

The primers and probes for IL-12, IFN-γ, iNOS, TNF–α, and glucose 6 phosphate dehydrogenase (G6PDH) were synthesized at the Sheldon Biotechnology Center (Montreal, Quebec) from previously published sequences. The nucleotide sequence of the primers and the probes used in these experiments are shown in Table I.

Total RNA isolation

Untreated and Poly I:C treated pregnant CBA/J females were sacrificed at day 8 of gestation, and individual embryos together with implantation sites were detached from the uterine wall and snap frozen in liquid nitrogen for subsequent RNA extraction. The whole implantation site was used as the source of total RNA for these studies due to difficulties in separating the embryo from the deciduum.

Isolation of total RNA was done according to the method developed by Chomczynski and Sacchi (20). Briefly, individual implantation sites were homogenized in 1ml of Trizol reagent (GIBCO, Burlington, Ontario) using a Polytron power homogenizer (Tekmar, Ohio, USA). Two hundred µl of chloroform was added and the samples were shaken vigorously for 15 seconds and then centrifuged for 15 minutes at 12,000g at 4 °C . Following centrifugation, the mixture had separated into a phenol-chloroform phase, an interface, and an aqueous phase containing the RNA. RNA is then precipitated from the aqueous fraction by mixing with isopropyl alcohol (500 µl) and centrifugation for 10 minutes at 4 °C. The RNA pellets were resuspended and washed once with 75% ethanol (1 ml) and pelleted by spinning the mixtures for 5 minutes at 4 °C. At the end of this procedure, the RNA pellets were air dried and then dissolved in RN-ase free water. The concentration of RNA in the final solution was

calculated from spectrphotometric readings at 260 nm wavelength. The RNA solution was diluted to a final concentration of 1μg/μl and stored at -80 °C.

Reverse transcriptase -Polymerase Chain Reaction (RT-PCR) analysis of IL-12, IFN-γ, iNOS, TNF-α and G6PDH mRNAs

One μg of total RNA was diluted in 10 μl of RN-ase free water and incubated at 65 °C for 5 minutes, and then added to the reverse transcription reaction mixture containing 1 μl of 0.1 M DTT, 4 μl of 5X single strand buffer (GIBCO, Burlington, Ontario), 2 μl of 10 mM dNTP mix, 1 μl containing 40 units of RNase inhibitor (Pharmacia), 1 μl of 100 nM of random hexonucleotide primers and 1 μl of RT solution containing 200 units of M-MLV RT (GIBCO, Burlington, Ontario). Following 1 hour incubation at 37 °C, the cDNA product was diluted 1:8, heated to 95 °C for 10 minutes and stored at -20 °C. Amplification by PCR was done by adding 10 μl of the cDNA synthesized in the reaction described above to 5 μl of 10X Taq Buffer (10mM Tris-HCl, 1.5 mM $MgCl_2$, and 50 mM KCl) (Boehringer Mannheim, Germany), and 1 μl of 10 pM of both sense and anti-sense primers specific for the cDNA to be amplified. The mixture was heated for 5 minutes at 95 °C before the addition of 2.5 units of Taq polymerase in 0.5 μl (Boehringer Mannheim, Germany). PCR

amplification proceeded according to the following amplification program: denaturation at 94 °C for 30 seconds; annealing at 58 °C for 1 minute and 30 seconds; and extension at 72 °C for 40 seconds for 30 cycles followed by a final extension step at 72 °C for 10 minutes. The PCR products were then stored at -20 °C. Fifteen µl of the amplified products were separated by agarose electrophoresis on 1% agarose in TBE buffer (0.089M Tris, 0.089M TBE Boric acid, $2X10^{-4}$ EDTA) containing ethidium bromide. The gel was washed in 0.25 N HCl for 5 minutes, 20 minutes in denaturing solution (4 g NaOH and 18 g NaCl in 200 ml of H_2O), and 20 minutes in neutralizing solution (36g NaCl and 200 ml of 1M Tris-HCl in 400 ml of H_2O) before being transferred under pressure onto a nylon membrane (Amersham). The DNA was cross-linked to the membranes by baking in a vacuum oven for 2 hours and stored at room temperature prior to hybridization.

Probe labeling and Southern blotting

The oligonucleotide probe (1 µl of 100 pmoles) was added to a mixture containing 1 µl of T4 polynucleotide Kinase (Pharmacia), 1 µl of 10X Kinase buffer (Pharmacia), and 5 µl of γ-^{32}P-ATP (Dupont). The mixture was incubated for 1-2 hours at 37 °C, followed by 10-20 minutes incubation at 65 °C to inactivate the T4 Kinase. Eighty µl of TE

buffer was added and the mixture was loaded onto a 5 ml Spin-column prepared from glass wool and Sephadex G50 in TE buffer to remove unbound γ-^{32}P-ATP. The column was centrifuged for 2 minutes and the eluate containing the ^{32}P labeled probe was quantified in a β counter. The membranes were first washed for 2-5 hours at 42 °C with 10 ml of a prehybridization solution containing 2.5 ml of 20X SSC (3 M NaCl, 0.3 M Na Citrate), 2 ml of 50 % Denhardt solution (1% of Ficoll, PVP, and BSA), 0.5 ml of 1 M $NaPO4^-$, 3.5 ml of 20% SDS, and 100 µl of 100 µg/ml Salmon sperm DNA (SSDNA) (Pharmacia). The membranes were then incubated overnight at 42 °C with the hybridization solution which contained 5 X 10^6 cpm of labeled probe diluted in the prehybridization buffer. The membranes were washed with 20% SSC and SDS and then the bands were visualized and quantified by phosphorimaging using a Molecular Dynamics PhosPhorimager with ImageQuaNT 4.1 software.

Results

Characterization and identification of GKO mice

The identity of homozygous GKO (gko/gko) and heterozygous (gko/-) mice was confirmed by screening tail biopsy DNA by PCR using specific primers supplied by Genentech. It is known that IFN–γ primes

macrophages to produce NO. In order to demonstrate that IFN–γ genes were functionally defective in GKO mice, homozygous and heterozygous mice were injected with alveolar hydatid cysts. This treatment normally induces IFN–γ production and subsequent macrophage priming (34). Peritoneal cells from acutely infected mice were collected 7 days later and challenged with 5 μg/ml LPS for 18 hours in a 5% CO_2 incubator at 37 °C. The results displayed in fig.1 show that peritoneal macrophages from the heterozygous control mice were primed and produced large amounts of nitrite when incubated with LPS as a secondary triggering signal. On the other hand, cells taken from gko/gko mice, that lack IFN-γ which serves as the primary signal, produced undetectable levels of nitric oxide after culture with LPS. This confirmed that GKO mice have functionally defective IFN–γ genes and their macrophages could not be induced to produce NO. We further observed that peritoneal cells from chronically infected (>3 post infection) homozygous gko/gko mice were able to produce significant levels of nitrite following incubation with LPS *in vitro* (data not shown). This indicated that in chronic infections, priming of macrophages could be induced by mediators other than IFN-γ. However, this has little relevance to the acute priming of decidual macrophages that accompanies implantation.

GKO mice are resistant to LPS induced embryo resorption

LPS is a powerful abortifacient factor which is known to induce abortion in a variety of mouse strains and other species (4,11,30,32). In this study, pregnant gko/gko and (gko/-) females were injected intravenously with 10 μg of LPS at day 7 of gestation as a source of exogenous triggering signal for macrophage activation. The mice were sacrificed at day 12 and the incidence of embryo loss was determined. The results presented in Table II indicated that gko/gko mice treated with LPS showed a low incidence loss of 12%. Interestingly, heterozygous females were sensitive to LPS and showed the expected incidence of embryo loss. These results suggested that in the absence of IFN-γ, a macrophage priming signal, GKO mice were resistant to LPS induced resorption indicating that the expression of IFN–γ was an essential early step in LPS induced embryo loss. Further, this data showed that LPS itself was not directly toxic to the growing embryos. This was further confirmed in LPS-non responsive C3H/HeJ mice which also showed resistance to LPS induce embryo loss without obvious signs of embryo pathology (data not shown).

Anti-IFN–γ antibodies reduced spontaneous early embryo loss

To further confirm the primary role of IFN–γ in early embryo loss, CBA/J females mated with DBA/2 males were injected intravenously with anti-IFN–γ antibodies or an isotype control antibody at day 6 of gestation. Table III shows that the incidence of embryo loss for mice injected with anti-IFN–γ was significantly reduced from 67% ($P<0.001$). These results confirmed the defect in priming in gko/gko mice and suggested that depletion of IFN–γ as early as day 6 augmented embryo survival.

Poly I:C Induced Embryo Loss is dependent on the Presence of IFN-γ.

Treatment of gravid mice with Poly I:C has also been shown to significantly increase embryo loss in most mouse strains (2,31). However, the mechanisms by which Poly I:C induced embryo loss are not fully understood, but many involve activated NK cells which are capable of IFN-γ production. In this study, we investigated whether Poly I:C induce embryo loss was conditional on the presence of IFN-γ. The results showed that gko/gko mice were significantly more resistant to Poly I:C treatments than heterozygous control mice (table IV). This

indicated that the mechanisms of Poly I:C induced embryo loss depended on the presence of IFN-γ. The results further showed a significant increase in the incidence of embryo loss in homozygous gko/gko mice when compared to homozygous mice that were not treated with Poly I:C. This suggested that the mechanisms of Poly I:C induced macrophage priming and embryo abortion might, in part, be mediated by factors other than IFN–γ as previously suggested by the data obtained from mice chronically infected by the parasite.

IFN–γ mRNA expression is associated with markers of embryo resorption

We have previously shown that at day 8 of gestation, decidual macrophages were activated as indicated by the expression of the inducible nitric oxide synthase (iNOS). In this study we investigated whether macrophage activation and nitric oxide production were the results of IFN-γ production. To investigate this hypothesis, we determined the expression of IFN–γ mRNA in the same embryos that simultaneously showed TNF–α and iNOS mRNAs expression. Total RNA was isolated from individual implantation sites of the CBA/J X DBA/2 pregnancies and specific mRNAs were amplified by RT-PCR using

primers specific for IFN–γ, TNF-α, iNOS and an internal control, glucose 6 phosphate dehydrogenase (G6PDH). After Southern blotting, ^{32}P labeled bands were detected by phosphorimaging. The results presented in figure 2 showed that implantation sites expressing iNOS mRNA, a marker of macrophage activation, also showed expression of IFN–γ mRNA. This suggested that IFN–γ expression was casually associated with decidual macrophage activation and NO production. Occasionally, we observed that some implantation sites showed mRNA expression of one of the cytokines but not of the others.

Collectively, these results showed that IFN-γ expression was needed as a primary signal to induce decidual macrophage priming. The results further showed that a secondary signal, was also essential for effecting early embryo loss. In this study, we also investigated the role of TNF-α as a potential secondary signal for the triggering of primed decidual macrophages *in utero*. The results in figure 2 showed that, in most cases, embryos that expressed iNOS and IFN-γ mRNAs also showed TNF-α expression. We can not exclude the possibility that TNF-α could mediate direct cytotoxic effect to target embryos.

The presence of IFN–γ is associated with IL-12 mRNA expression

It has been reported that the presence of IL-12 (16) enhanced the induction of IFN–γ production, and diminished the inhibitory effect of TH2 cytokines on IFN-γ induction. In this study, we investigated whether the expression of IFN–γ mRNA in potentially resorbing embryos was associated with the local production of IL-12. We performed RT-PCR on individual embryos using primers specific for IL-12, IFN–γ, TNF–α, and iNOS. The results presented in fig (3) showed that the expression of IL-12 was associated, in most cases, with TH1 cytokine expression.

We do not believe that IL-12 was solely responsible for the expression of IFN-γ mRNA and consequent macrophage activation because some implantation sites that showed IFN-γ mRNA expression but lacked IL-12 expression (fig 3). Furthermore, data obtained from studying a greater number of mice showed that the majority of implantation sites simultaneously expressed IFN-γ and IL-12 mRNA with macrophage activation markers respectively (data not shown).

Discussion:

Early embryo loss is considered one of the most common complications of mammalian pregnancies. It may be defined as losses that occur before the third week of human gestation and often takes place without being clinically observed. In murine reproduction, CBA/J

female mated with DBA/2 male provides a model for early embryo loss which has been studied extensively to discover early cellular and molecular events associated with early embryo loss (8).

In earlier reports, we showed that NK cells and activated macrophages were involved in embryo resorption. Mice that were treated with agents that augmented NK cells activity, such as Poly I:C or IL-2, resorbed at a much higher rate than untreated mice (2,9). On the other hand, treatments that abrogated NK activity were advantageous for embryo survival (31). More recently, we have shown that treatments that reduced the number of macrophages increased embryo survival (10). However, it is not known which of these cells mediate direct damage to the embryo *in vivo*. It would appear unlikely that NK cells, through secretion of cytotoxic molecules, were directly responsible for embryo loss, since NK cells isolated from early implantation sites showed minimal cytotoxicity *in vitro* (Baines, unpublished data). Further, there was no difference in perforin expression in decidual NK cells from resorbing compared to non-resorbing embryos (26,33) and (Haddad, unpublished data). On the other hand, recent experiments showed that activated decidual macrophages were capable of directly mediating embryo damage through the production of nitric oxide (23). Inhibition of NO production alone significantly enhanced embryo survival (23). Therefore, NK cells may be only indirectly involved in facilitating early

embryo loss through the production of cytokines such as IFN-γ and TNF-α (18,24).

IFN–γ is considered to be the major factor responsible for macrophage activation. In this study, we investigated the role of IFN–γ in early embryo loss. The data showed that the incidence of both LPS and Poly I:C induced embryo loss in IFN–γ-deficient mice was significantly lower than that for control mice. In addition, normal CBA mice given anti-IFN–γ antibody also showed a reduced incidence of embryo resorption. These results suggested that IFN–γ was required in the early events that lead to embryo resorption. The results also suggested that LPS was not embryo-toxic to the growing embryos since gko/gko and LPS non-responsive mice (data not shown) showed normal rate of embryo survival.

We have recently reported that both primary and triggering signals were needed for nitric oxide production by activated decidual macrophages in vitro (23). Therefore, we hypothesized that uterine IFN–γ produced by decidual NK cells primes resting decidual macrophages. A second signal such as TNF–α produced by NK cells or primed macrophages could then trigger macrophage activation for nitric oxide production and cause subsequent embryo resorption. Hence, in the absence of an effective priming signal (IFN–γ deficient mice, and anti-IFN–γ antibody treated mice), there is no nitric oxide production and

embryo resorption is abrogated. We can not exclude the possibility that TNF-α produced by either NK cells or macrophages would mediate cytotoxicity to the embryonic cells, however in all cases, the mere presence of TNF-α would indicate its availability to primed decidual macrophages to trigger NO production.

We have previously shown that nitric oxide, produced by activated decidual macrophages, mediated embryo resorption (23). In this study, we investigated whether macrophage activation, nitric oxide production, and embryo loss were associated with increased IFN–γ mRNA expression. Our results showed that iNOS and TNF–α mRNAs were simultaneously expressed in the same embryos that showed IFN–γ mRNA expression. This is in agreement with our proposed hypothesis that IFN–γ activates macrophages as indicated by the upregulation of the two macrophage activation markers, TNF–α and iNOS, which are directly associated with embryo loss.

This interpretation was supported by the observation that IFN-γ deficient mice were significantly more resistant to both LPS and Poly I:C induced abortion than wild type mice. This suggested that the mechanism of Poly I:C induced embryo abortion may be, in great part, due to the increased production of IFN-γ by Poly I:C activated NK cells. The IFN-γ might then prime decidual macrophages for nitric oxide production and embryo death.

While a viral infection may induce embryo loss as a consequence of interferon induction, spontaneous embryo loss in normal CBA/J mice suggests an endogenous factor. Therefore, we investigated the possible involvement of the NK cell activating cytokine, IL-12, in early embryo loss. It is known that IL-12 plays a fundamental role in regulating immune responses, by favoring Th1 cytokine production. In this study, we investigated whether the expression of IFN–γ mRNA in potentially resorbing embryos was associated with IL-12 mRNA expression. Single embryo studies using RT-PCR showed that IL-12 expression was detected in the same embryos that also showed TNF–α, IFN–γ, and iNOS mRNAs expression. This suggested that IL-12 could be involved in IFN–γ expression and subsequent nitric oxide production and embryo death. The results further showed that some implantation sites lacked correlation in the expression of IL-12 and IFN-γ mRNA. This might be due to the involvement of factors other than IL-12 in mediating the expression of IFN-γ mRNA.

In summary, the results showed that decidual macrophage activation and embryo abortion in mice was dependent upon the presence of both primary and triggering signals in decidual cells. Further, the results presented in this paper clearly demonstrated that IFN–γ production was crucial for macrophage activation and LPS and Poly I:C induced embryo loss. The simultaneous expression of IL-12 and

IFN-γ mRNAs in resorbing embryos suggested that the increased expression of IFN-γ might have been due to increased IL-12 expression.

Figure Legends:

Figure 1: Production of nitric oxide by peritoneal macrophages of IFN-γ knockout mice (GKO) and heterozygous control mice. Six gko/gko and six control mice were injected with 50 alveolar hydatid cyst larvae. Seven days later, peritoneal macrophages were collected and challenged for 18 hours with 5 μg/ml of LPS *in vitro*. Cell supernatants were assayed for the presence of nitric oxide using the Greiss reaction. Black bars represent cells treated with LPS, whereas white bars indicate cells treated with control medium.

Figure 2: Representative pregnancy showing the Expression of IFN-γ, TNF-α, and iNOS mRNA in individual embryos of CBA/J females mated with DBA/2 males. Total RNA was isolated from individual implantation sites of 5 Poly I:C treated (60 μg) CBA/J females. Total RNA was reverse transcribed and PCR amplified using primers specific for IFN-γ, TNF-α, and iNOS. The samples were separated by 1% agarose gel electrophoresis and transferred to nylon membranes, which were hybridized with ^{32}P labeled specific probes. The membranes were visualized and quantitated using PhosPhorimager densitometry relative to the G6PDH housekeeping gene. Solid bars indicate IFN-γ expression,

open bars indicate TNF-α expression and the hatched bars indicate iNOS expression.

Figure 3. Representative data showing the correlation of IL-12 mRNA expression with markers of macrophage activation and embryo resorption. Six normal CBA/J females mated with DBA/2 males were killed at day 8 of gestation. Expression of IL-12, IFN-γ, TNF-α , and iNOS mRNAs were analyzed as discussed in figure 2. Crossed bars indicate (IL-12 expression), solid bars indicate (IFN-γ expression), open bars indicate (TNF-α expression), and horizontal bars indicate (iNOS expression).

Bibliography

1. Clark, D. A. and G. Chaouat. 1989. What do we know about spontaneous abortion mechanisms? *Am. J. Reprod. Immunol.* 19:28.-37.

2. Kinsky, R. G., G. Delage, N. Rosin, M. N. Thang, M. Hoffmann, and G. Chaouat. 1990. A murine model of NK cell mediated resorption. *Am. J. Reprod. Immunol.* 23:73.-77.

3. Duclos, A. J., E. K. Haddad, and M. G. Baines. 1995. Presence of activated macrophages in a murine model of early embryo loss. *Am. J. Reprod. Immunol.* 33:354.-366.

4. Baines, M. G., A. J. Duclos, A. R. Defougerolles, and R. L. Gendron. 1996. Immunological prevention of spontaneous early embryo resorption is mediated by non-specific immunostimulation. *Am. J. Reprod. Immunol.* 35:34.-42.

5. Green, L. C., D. A. Wagner, J. Glogowski, P. L. Skipper, J. S. Wishnok, and S. R. Tannenbaum. 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Analytical Biochemistry* 126:131.-138.

6. Adams, D. O. and T. A. Hamilton. 1984. The cell biology of macrophage activation. *Ann. Rev. Immunol.* 2:283.-318.

7. Paul, W. E. and R. A. Seder. 1994. Lymphocyte responses and cytokines. [Review]. *Cell* 76:241.-251.

8. Clark, D. A., M. R. McDermott, and M. R. Szewczuk. 1980. Impairment of host-versus-graft reaction in pregnant mice. II. Selective suppression of cytotoxic T-cell generation correlates with soluble suppressor activity and with successful allogeneic pregnancy. *Cell. Immunol.* 52:106.-118.

9. Gendron, R. L. and M. G. Baines. 1988. Infiltrating decidual natural killer cells are associated with spontaneous abortion in mice. *Cell. Immunol.* 113:261.-267.

10. Duclos, A. J., D. K. Pomerantz, and M. G. Baines. 1994. Relationship between decidual leukocyte infiltration and spontaneous abortion in a murine model of early fetal resorption. *Cell. Immunol.* 159:184.-193.

11. Chaouat, G. 1994. Synergy of lipopolysaccharide and inflammatory cytokines in murine pregnancy: alloimmunization prevents abortion but does not affect the induction of preterm delivery. *Cell. Immunol.* 157:328.-340.

12. Chaouat, G., E. Menu, C. Bonneton, and R. G. Kinsky. 1989. Immunological manipulations in animal pregnancy and models of pregnancy failure. *Curr. Opin. Immunol.* 1:1153.-1156.

13. Wegmann, T. G. 1990. The cytokine basis for cross-talk between the maternal immune and reproductive systems. *Curr. Opin. Immunol.* 2:765.-769.

14. Munoz-Fernandez, M. A., M. A. Fernandez, and M. Fresno. 1992. Synergism between tumor necrosis factor-alpha and interferon-gamma on macrophage activation for the killing of intracellular Trypanosoma cruzi through a nitric oxide-dependent mechanism. *Eur. J. Immunol.* 22:301.-307.

15. Duncan, D. D. and S. L. Swain. 1994. Role of antigen-presenting cells in the polarized development of helper T cell subsets: evidence for

differential cytokine production by Th0 cells in response to antigen presentation by B cells and macrophages. *Eur. J. Immunol.* 24:2506.-2514.

16. Micallef, M. J., T. Ohtsuki, K. Kohno, F. Tanabe, S. Ushio, M. Namba, T. Tanimoto, K. Torigoe, M. Fujii, M. Ikeda, S. Fukuda, and M. Kurimoto. 1996. Interferon-gamma-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon-gamma production. *Eur. J. Immunol.* 26:1647.-1651.

17. Trinchieri, G. and B. Perussia. 1985. Immune interferon: A pleiotropic lymphokine with multiple effects. *Immunol. Today* 6:131.-136.

18. Weigent, D. A., G. J. Stanton, and H. M. Johnson. 1983. Interleukin 2 enhances natural killer cell activity through induction of gamma interferon. *Infect. Immun.* 41:992.-997.

19. Vilcek, J. and I. C. Oliveira. 1994. Recent progress in the elucidation of interferon-gamma actions: molecular biology and biological functions. [Review]. *International Archives of Allergy & Immunology* 104:311.-316.

20. Chomczynski, P. and N. Sacchi. 1987. Single Step Method of RNA Isolation by Acid Guanidinium Thiocyanate Phenol Chloroform Extraction. *J. Anal. Biochem.* 162:156.-159.

21. Chan, S. H., B. Perussia, J. W. Gupta, M. Kobayashi, M. Pospisil, H. A. Young, S. F. Wolf, D. Young, S. C. Clark, and G. Trinchieri. 1991. Induction of IFN-gamma production by NK cell stimulatory factor and synergy with other inducers. *J. Exp. Med.* 173:869.-879.

22. D'Andrea, A. D., M. Rengaraju, N. M. Valiante, J. Chehimi, M. Kubin, M. Aste, S. H. Chan, M. Kobayashi, D. Young, E. Nickbarg, R. Chizzonite, S. F. Wolf, and G. Trinchieri. 1992. Production of NKSF (IL-12) by peripheral blood mononuclear cells. *J. Exp. Med.* 176:1387.-1398.

23. Haddad, E. K., A. J. Duclos, and M. G. Baines. 1995. Early embryo loss is associated with local production of nitric oxide by decidual mononuclear cells. *J. Exp. Med.* 182:1143.-1151.

24. Handa, K., R. Suzuki, H. Matsui, Y. Shimizu, and K. Kumagai. 1983. NK cells as a responder to IL-2: II. IL-2 induced IFN-γ production. *J. Immunol.* 130:988.-992.

25. Ding, A. H., C. F. Nathan, and D. J. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol.* 141:2407.-2412.

26. Parr, E. L., L. H. Young, M. B. Parr, and J. D. E. Young. 1990. Granulated metrial gland cells of pregnant mouse uterus are natural killer-like cells that contain perforin and serine esterases. *J. Immunol.* 145:2365.-2372.

27. Tangri, S., T. G. Wegmann, H. Lin, and R. Raghupathy. 1994. Maternal anti-placental reactivity in natural, immunologically-mediated fetal resorptions. *J. Immunol.* 152:4903.-4911.

28. Krishnan, L., L. J. Guilbert, T. G. Wegmann, M. Belosevic, and T. R. Mosmann. 1996. T helper 1 response against *Leishmania major* in pregnant C57bl/6 mice increases implantation failure and fetal resorptions -correlation with increased IFN-γ and TNF-α and reduced IL-10 production by placental cells. *J. Immunol.* 156:653.-662.

29. Chaouat, G., E. Menu, D. A. Clark, M. Dy, M. Minkowski, and T. G. Wegmann. 1990. Control of fetal survival in CBA x DBA/2 mice by lymphokine therapy. *J. Reprod. Fert.* 89:447.-458.

30. Gendron, R. L., F. P. Nestel, W. S. Lapp, and M. G. Baines. 1990. Lipopolysaccharide-induced fetal resorption in mice is associated with the intrauterine production of tumour necrosis factor-alpha. *J. Reprod. Fert.* 90:395.-402.

31. de Fougerolles, A. R. and M. G. Baines. 1987. Modulation of the natural killer cell activity in pregnant mice alters the spontaneous abortion rate. *J. Reprod. Immunol.* 11:147.-153.

32. Clark, D. A., D. K. Banwatt, and G. Chaouat. 1993. Effect of prostaglandin synthesis inhibitors on spontaneous and endotoxin-induced abortion in mice. *J. Reprod. Immunol.* 24:29.-44.

33. Parr, E. L., H. L. Chen, M. B. Parr, and J. S. Hunt. 1995. Synthesis and granular localization of tumor necrosis factor-alpha in activated NK cells in the pregnant mouse uterus. *J. Reprod. Immunol.* 28:31.-40.

34. Alikhan, Z., W. Li, and S. L. Chan. 1996. Animal model for the pathogenesis of reactive amyloidosis [Review]. *Parasitology Today* 12:297.-302.

Fig. 1 Production of Nitric Oxide by Peritoneal Macrophages from Control and GKO Mice

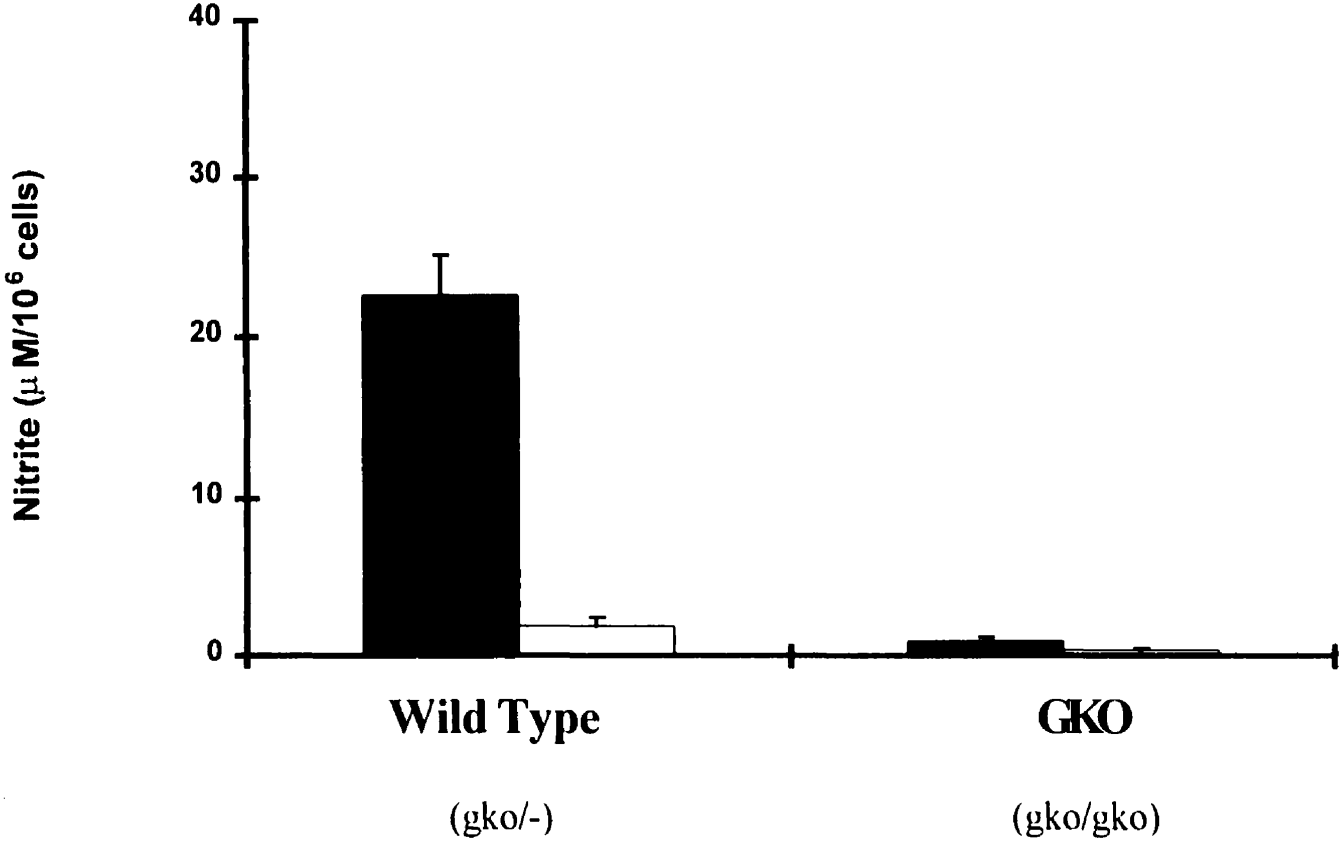

Fig. 2 Expression of IFN-γ,TNF-α, and iNOS mRNAs in Individual Implantation Sites of Pregnant CBA/J Females Mated by DBA/2 Males

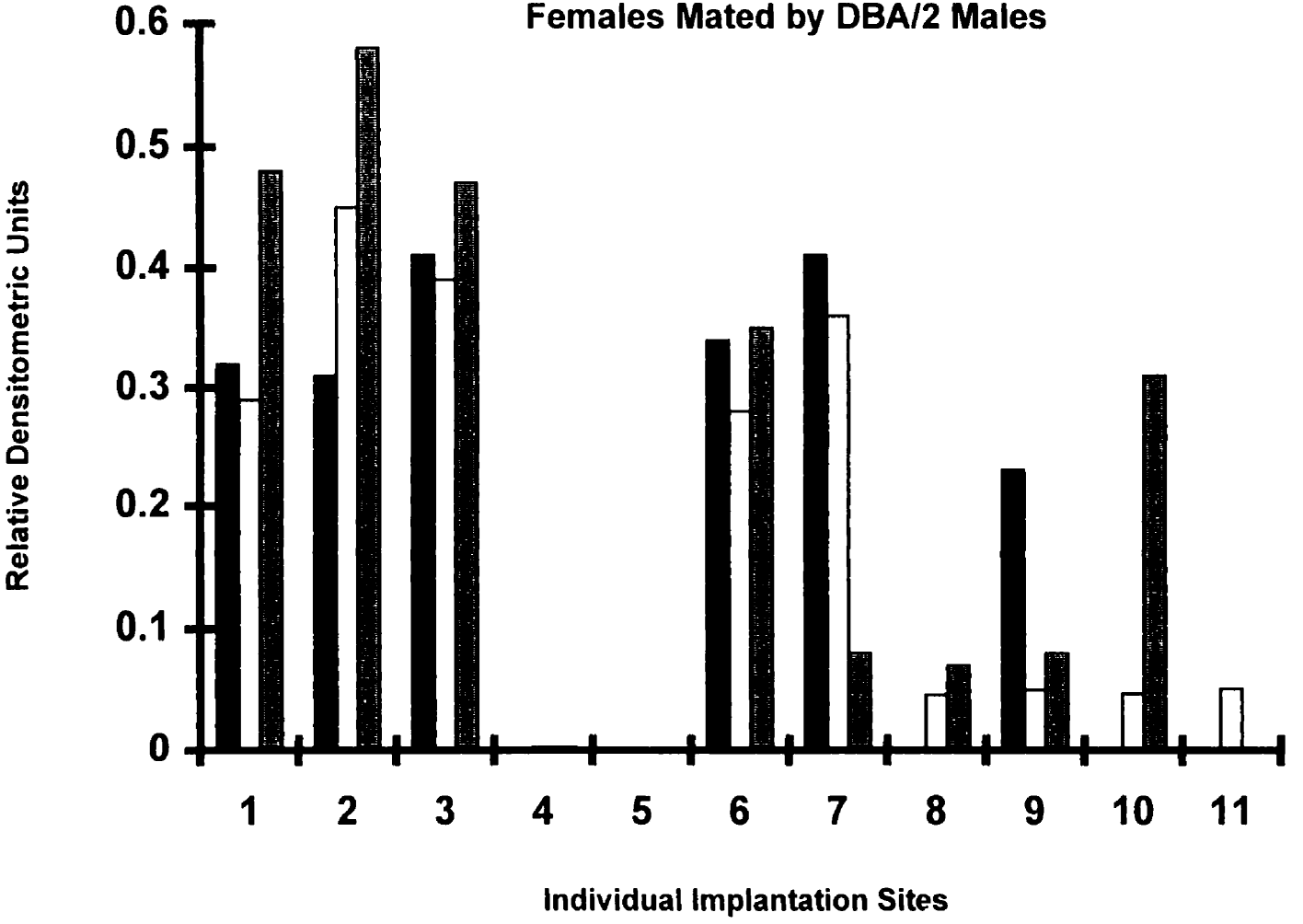

Fig. 3 Association of IL-12 mRNA Expression with Markers of Macrophage Activation

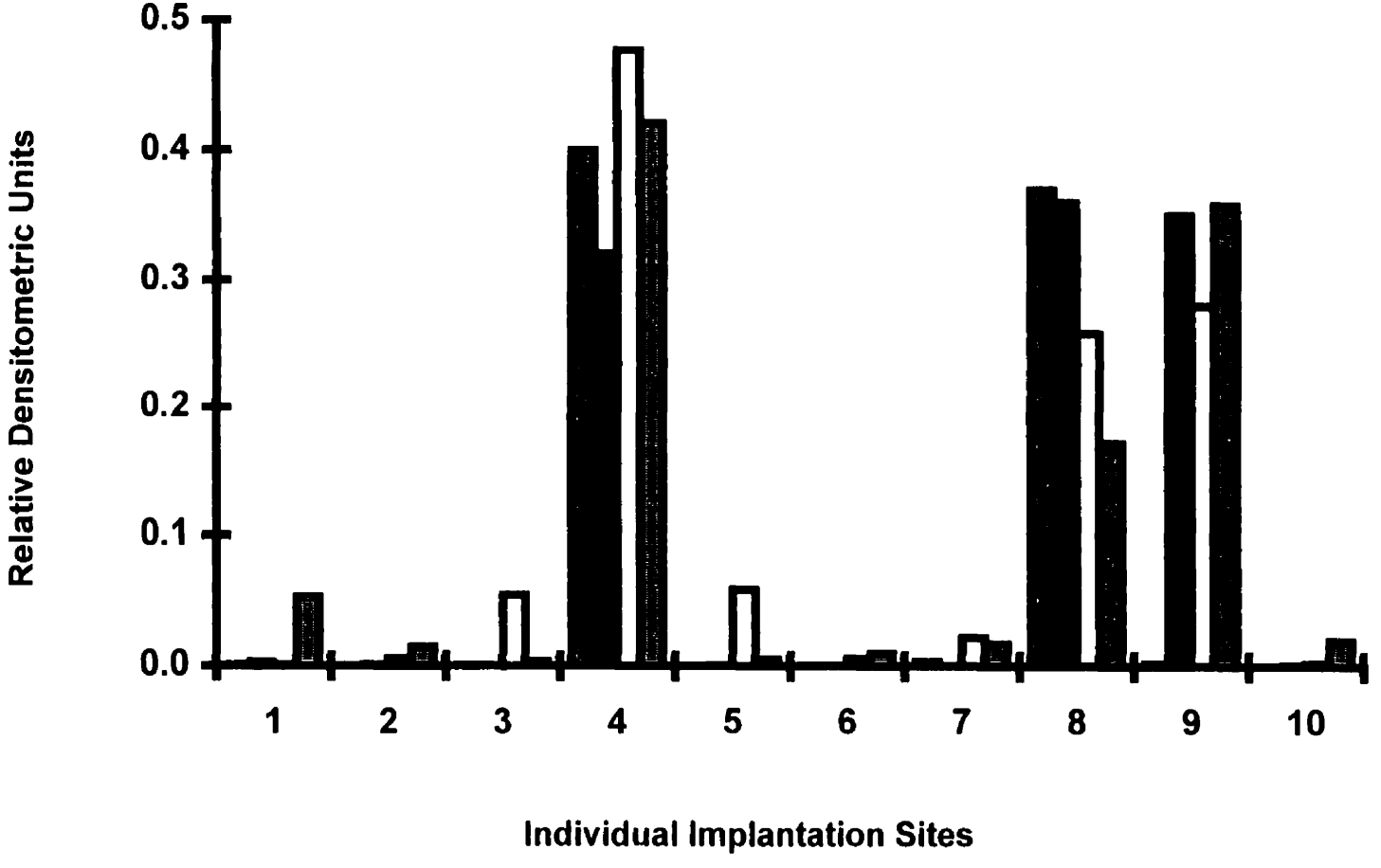

Table I. Primer and Probe for IFN-γ, IL-12, TNF-α, iNOS, and G6PDH Sequences

Specificities		Oligonucleotide Sequences	Expected PCR Product (bp)
iNOS	Sense	GAGCCTCGTGGCTTTGGGCTCCTC	486
	Antisense	GCGACGACGGTCTTTGAAGCCTTC	
	Probe	ACGTTCAGCACATCCTGCAAAAGCAGCTGC	
TNF-α	Sense	CCAGACCCTCACACTCAGAT	498
	Antisense	AACACCCATTCCCTTCACAG	
	Probe	CCAAGTGGAGGAGCAGCTGGAG	
TNF-γ	Sense	GCTGTTTCTGGCTGTTACTG	426
	Antisense	AATCAGCAGCGACTCCTTTTCC	
	Probe	GACGAACTGGCAAAAGGATGG	
IL-12 (p40)	Sense	CGTGCTCATGGCTGGTGCAAAG	313
	Antisense	CTTCATCTGCAAGTTCTTGGGC	
	Probe	TCTGTCTGCAGAGAAGGTCACA	
G6PDH	Sense	CTAAACTCAGAAAACATCATGGC	111
	Antisense	GGAAGGTGGTTCGACTATGTG	
	Probe	GAGCAGGTGGCCCTGAGCCG	

Table II. GKO mice are resistant to LPS induced embryo loss

Experimental groups	Number of Mice tested	Implantation sites/mouse	Number of Resorbing Embryos	Percent of Embryo loss
gko/gko mice	7	11.0± 2.0	9	11.7%
gko/- mice	6	9.3 ± 1.4	26	46.5%

Pregnant gko/gko and heterozygous control mice (gko/-) were injected with 10 μg of LPS intravenously at day 7 of gestation. At day 12 of gestation, the mice were sacrificed and the number of resorbing and non-resorbing embryos were counted.The incidence of embryo loss was calculated using the formula presented in the materials and methods.

Table III. Passive Anti-IFN-γ antibodies reduce spontaneous embryo resorption in CBA/J X DBA/2 pregnancies

Experimental groups	Number of Mice tested	Implantation sites/mouse	Number of Resorbing Embryos	Percent of Embryo loss
XMG1.2 treated CBA/J	4	9.2± 0.8	4	8.4%*
Control antibody treated CBA/J	5	8.8 ± 1.0	9	25.6%

* $P < 0.001$ compared to control using student t-test.

Pregnant CBA/J females mated with DBA/2 males received an intraperitonel injection of 100 μl of XMG1.2, anti-IFN-γ antibodies at day 6 of gestation. A control group of mice were injected with the same amount of an isotype matched control antibody. Mice were killed at day 12 of gestation and the incidence of embryo loss was calculated.

Table IV. Poly I:C Induced Early Embryo Loss is Dependent on the Presence of IFN-γ

Experimental groups	Number of Mice tested	Implantation sites/mouse	Number of resorbing embryos	Percent of resorbing embryos
Untreated gko/gko	8	11.2 ± 1.4	2	2.3%
Poly I:C treated gko/gko mice	9	10.2 ± 0.9	15	17.6%
Poly I:C treated (gko/-) Mice	8	10.6± 1.1	71	77.2%

Pregnant mice were intravenously injected with 100μg of Poly I:C at day 7 of gestation. The mice were killed at day 12 of gestation, and the incidence of early embryo loss was calculated.

Preface to chapter 4

In this study, we investigated the role of TNF-α as a second signal needed for macrophage activation and embryo resorption. Therefore, we studied the expression of TNF-α mRNA in individual embryos of the spontaneous and poly I:C induced models of early embryo loss.

Early Embryo Loss is Associated with the Prior Expression of Macrophage Activation Markers in the Decidua[1]

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Abstract:

In early embryo loss, the activation of maternal immune effector mechanisms play a critical role in determining the success or failure of a pregnancy. We have previously shown that increased nitric oxide (NO) production by decidual macrophages is involved in early embryo loss occurring at day 12 of gestation. In this study, using RT-PCR and southern blotting, the expression of iNOS and TNF-α mRNA was determined to quantify macrophage activation in individual murine embryos in a model of spontaneous early embryo loss. At day 8 of gestation, 32% and 29% of embryos with no apparent pathology showed an increase in iNOS and TNF-α mRNA expression respectively. This corresponds to the natural resorption rate seen in the mouse model. In addition, the percentage of embryos with increased iNOS and TNF-α mRNA expression was further augmented when pregnant mice were induced to abort at a higher rate. These results showed, for the first time, a correlation between increased iNOS and TNF-α expression and embryo resorption. The results provide evidence for the presence of activated macrophages at implantation sites before overt embryo damage occurs.

Introduction:

In humans, early embryo loss may be defined as losses that occur before the third week of gestation. Hence, in humans, most pregnancies terminate before the onset of the second menstrual cycle and occur unnoticed. To characterize the cellular events associated with early embryo loss, we have studied a murine model in which 20%-30% of the embryos spontaneously resorb by day 12 of gestation (1). The resorption rate can be further increased 2-3 fold if pregnant mice are treated with a double stranded RNAs that induce interferon production and enhance natural killer (NK) cell activity (2,3). The mechanism of enhancement of early embryo loss by poly innosinic: cytidylic acid (poly I:C) showed a similar time course and cytopathology to that observed for spontaneous embryo loss in untreated CBA/J females mated with DBA/2 males (4,5). Using this model, several laboratories showed that early embryo loss was associated with the activation of non-specific immune mechanisms (6). Anti-Asialo-GM1, a rabbit anti-NK cell antiserum, significantly reduced the incidence of embryo resorption (2). Moreover, immunohistochemical analysis performed on individual implantation sites, showed that NK cells heavily infiltrated some, but not all embryos at day 8 of gestation (7). Likewise, macrophages which constitute another population of non-specific effector cells were also shown to be

significantly elevated in resorbing compared to non-resorbing embryos (4). Most of these macrophages were found at the decidual tissues of implantation sites and were not found in the embryo itself (4). Taken together, these results suggest a role for both NK cells and macrophages in early embryo loss (5).

Recently, we showed that nitric oxide (NO), a cytotoxic molecule produced by activated macrophages, is directly responsible for early embryo loss (8). Nitric oxide is a short-lived pleiotropic mediator which is synthesized from L-arginine by constitutive or inducible nitric oxide synthases (NOS) (9-11). Of the three isotypes of the NOS enzyme which have been identified to date, the inducible NOS (iNOS) is present almost exclusively in activated macrophages and neutrophils (12). The iNOS is inducible by IFN-γ, TNF–α, Interleukin-1 (IL-1), and LPS (13,14). Nitric Oxide synthases can be inhibited by many L-arginine analogues such as N-monomethyl arginine (L-NMMA), and aminoguanidine (AG) (15,16). The latter is much more inhibitory to the iNOS rather than to the two constitutive NOS (17-19). Our previous results indicated that maternal macrophages at the implantation sites of 29% of the embryos produced significant amount of nitrite and nitrate, the oxidation products of NO. Significantly, embryo survival was dramatically enhanced when pregnant CBA/J mice were treated with aminoguanidine, indicating that NO alone was essential for the embryo loss observed (8).

Likewise TNF–α has also been shown to be involved in fetal resorption. TNF-α is a multifunctional cytokine produced by a variety of cell types. Depending on the stimulus and the cellular target, TNF-α has cytotoxic, cytostatic, immunomodulatory, growth promoting and many other activities (20). Exogenous TNF–α can induce abortion in the low abortion and high abortion matings (21,22). Furthermore, inhibition of TNF–α production by anti-TNF-α, or pentoxifylline increases embryo survival possibly by preventing the TNF-α mediated triggering of NO release (23).

Analysis of gene expression provides a more sensitive method for the detection of cell activation. The mechanism of iNOS mRNA induction in macrophages has been explored by cloning and sequencing the 5' promoter region of the mouse iNOS gene (24-26). This region was shown to contain binding consensus sequences for different nuclear factors including the nuclear factor of κB (NFκB) heterodimers (27). In addition, 10 copies of IFN–γ response elements (γ-IRE), as well as consensus sequences for interferon regulatory factor elements (IRF-E) have also been found upstream of the iNOS transcription initiation site (24,26,27). These findings outline some of the mechanisms by which iNOS mRNA expression is induced in response to different stimuli. Induction of TNF–α mRNA expression in macrophages has also been shown to be influenced by IFN–γ or LPS (28-30). Therefore, the

persistence and accumulation of both iNOS and TNF-α mRNA depend on the continuous presence of external stimuli, thus making the expression of iNOS and TNF-α mRNA a good indicator of macrophage activation (24,25). While TNF-α is also produced by NK cells and the presence of TNF-α could also be an indicator of NK cell activation, quantitatively the major source of TNF-α production is the macrophage (31,32).

The rationale behind these studies is that the priming of decidual macrophages occurs *in vivo* prior to any apparent embryo damage. In this paper, we show that the state of macrophage activation as indicated by the simultaneous expression of iNOS and TNF-α mRNA at the implantation sites of individual embryos correlates with the incidence of subsequent fetal wastage. These studies confirm at the molecular level the association of NO and TNF-α with early embryo loss.

Materials and Methods:

Animals and Matings

CBA/J females ($H2^k$) and Balb/c males ($H2^d$) were purchased from The Jackson Laboratory (Bar Harbor, Maine) at an age of 8 weeks. DBA/2 males ($H2^d$) were acquired from Charles River (St. Constant, Quebec).

The animals were allowed to adjust to the housing conditions for 6-8 weeks, and then allowed to mate. The housing and handling of the experimental animals was in accordance with the guidelines of the Canadian Council for Animal Care. Four CBA/J females were housed with a single DBA/2 or a single Balb/C male, and checked daily for the presence of a copulatory plug. The day of appearance of a mating plug was arbitrarily designated as day 0. In some, pregnant CBA/J females received intraperitoneal (i.p) injection of 60 μg of Poly I:C on day 7 to augment embryo losses (Sigma, St. Louis, MO). Treatment with poly I:C has been shown to increase fetal resorption to 60% (2). All pregnant females were sacrificed at day 8 of gestation, and individual embryos together with implantation sites were detached from the uterine wall and snap frozen in liquid nitrogen for subsequent RNA extraction. At day 8, both the embryo and the implantation sites were used as sources of total RNA due to difficulties in separating them.

Primers and Probes

The primers and probes for iNOS, TNF–α, and glucose 6 phosphate dehydrogenase (G6PDH) were synthesized at the Sheldon Biotechnology Center (Montreal, Quebec) from previously published

sequences. The nucleotide sequence of the primers and the probes are shown in Table I.

Total RNA Isolation

Isolation of total RNA was done according to the method developed by Chomczynski and Sacchi (33). Briefly, individual implantation sites were homogenized in 1ml of Trizol reagent (GIBCO, Burlington, Ontario) using a Polytron power homogenizer (Tekmar, Ohio, USA). The homogenized samples were incubated for 5 minutes at room temperature to allow the complete dissociation of nucleoprotein complexes. Two hundreds µl of chloroform was added and the samples were shaken vigorously for 15 seconds and then incubated for 2-3 minutes at room temperature. The samples were then centrifuged for 15 minutes at 12,000g at 4 °C . Following centrifugation, the mixture separated into a phenol-chloroform phase, an interphase, and an aqueous phase containing the RNA. RNA is then precipitated from the aqueous fraction by mixing with isopropyl alcohol (500 µl) and centrifugation for 10 minutes at 4 °C. After removing the supernatant, RNA pellets were resuspended and washed once with 75% ethanol (1 ml) by spinning the mixtures for 5 minutes at 4 °C. At the end of this procedure, the RNA pellets were air dried and then dissolved in RN–ase free water. The concentration of RNA in the final solution was calculated from the spectrphotometric readings at 260 nm wavelength. The RNA

solution was diluted to a final concentration of 1μg/μl and stored at -70 °C for further use.

Reverse Transcriptase (RT-PCR) analysis of iNOS, TNF-α and G6PDH mRNAs

The reverse transcription reaction was prepared from 1 μl of 0.1 M DTT, 4 μl of 5X single strand buffer (GIBCO, Burlington, Ontario), 2 μl of 10 mM dNTP mix, 1 μl containing 40 units of RNase inhibitor (Pharmacia), 1 μl of 100 nM of random hexonucleotides and 1 μl containing 200 units of M-MLV RT (GIBCO, Burlington, Ontario). One μg of total RNA was diluted in 10 μl of RN-ase free water and incubated at 65 °C for 5 minutes, and then added to the reaction mixture to give a total volume of 20 μl. After 1 hour incubation at 37 °C, the mixture was diluted 1:8, heated to 95 °C for 10 minutes and stored at -20 °C for PCR amplification. Amplification by PCR was done by adding 10 μl of the cDNA synthesized in the reaction described above to 5 μl of 10X Taq Buffer (10mM Tris-HCL, 1.5 mM $MgCl_2$, and 50 mM KCl) (Boehringer Mannheim, Germany), and 1 μl of 10 pM of both sense and anti-sense primers specific for the cDNA to be amplified. The mixture was heated for 5 minutes at 95 °C before the addition of 2.5 units of Taq polymerase in 0.5 μl (Boehringer Mannheim, Germany). PCR amplification proceeded according to the following amplification program: denaturation at 94 °C for 30 seconds; annealing at 58 °C for 1

minute and 30 seconds; and extension at 72 °C for 40 seconds for 30 cycles followed by a final extension step at 72 °C for 10 minutes. The PCR products were then stored at -20 °C for agarose electrophoresis. Fifteen µl of the amplified products were separated on 1% agarose in TBE buffer (0.089M Tris, 0.089M TBE Boric acid, $2X10^{-4}$ EDTA) containing ethidium bromide. The gel was washed in 0.25 N HCl for 5 minutes, 20 minutes in denaturing solution (4 g NaOH and 18 g NaCl in 200 ml of H_2O), and 20 minutes in neutralizing solution (36g NaCl and 200 ml of 1M Tris-HCl in 400 ml of H_2O) before being transferred under pressure onto a nylon membrane (Amersham). The DNA was cross-linked to the membranes by baking in a vacuum oven for 2 hours and stored at room temperature for future hybridization.

Probe Labeling and Southern Blotting

The oligonucleotide probe (1 µl of 100 pmoles) was added to a mixture containing 1 µl of T4 polynucleotide Kinase (Pharmacia), 1 µl of 10X Kinase buffer (Pharmacia), and 5 µl of γ-^{32}P-ATP (Dupont). The mixture was incubated for 1-2 hours at 37 °C, followed by 10-20 minutes incubation at 65 °C to inactivate the T4 Kinase. Eighty µl of TE buffer was added and the mixture was loaded onto a Spun column prepared from glass wool and Sephadex G50 in TE buffer to remove

unbound γ-^{32}P-ATP. The column was centrifuged for 2 minutes and the eluate containing the ^{32}P labeled probe was quantitated in a β counter. The membranes were first washed for 2-5 hours at 42 °C with 10 ml of a prehybridization solution containing 2.5 ml of 20X SSC (3 M NaCl, 0.3 M Na Citrate), 2 ml of 50 % Denhardt (1% of Ficoll, PVP, and BSA), 0.5 ml of 1 M NaPi, 3.5 ml of 20% SDS, and 100 µl of 100 µg/ml Salmon sperm DNA (SSDNA) (Pharmacia). The membranes were then incubated overnight at 42 °C with the hybridization solution which contained sufficient labeled probe diluted in the prehybridization buffer to give 5 X 10^6 cpm. The membranes were washed with 20% SSC and SDS and then the bands were visualized and quantified by phosphorimaging using a Molecular Dynamics PhosPhorimager with ImageQuaNT 4.1 software.

Results:

Association of iNOS mRNA expression with early embryo loss

We have previously shown that decidual NO production was associated with embryo resorption in low and high loss pregnancies. Furthermore, we showed, using immunohistochemistry, that the macrophages in the individual implantation sites express iNOS protein

at day 8 of gestation (8). In this study, we have investigated the expression of iNOS mRNA using RT-PCR and Southern blotting. Figure 1 shows a representative Southern analysis of individual implantation sites from a single untreated (fig. 1A) and Poly I:C treated (fig 1B) CBA/J x DBA/2 pregnancy. The basal level of iNOS mRNA expression was determined by calculating the upper 95% confidence limit (UCL) of iNOS mRNA expression in all embryos from low embryo loss matings of CBA/J X Balb/C at day 8 of gestation. Expression values that were higher that the UCL were considered significantly increased. As shown in fig. 1A, 2 out of 10 (20%) embryos showed a significant increase in iNOS mRNA expression. Likewise, increased iNOS mRNA expression was observed in 4 out of 7 (57%) embryos when pregnant females were treated with Poly I:C to augment the incidence of abortion (fig. 1B).

Association of TNF–α mRNA expression with early embryo loss

In these experiments, we examined the expression of TNF–α mRNA in individual embryos in relation to early embryo loss. Fig. 2 shows a representative Southern analysis of untreated (fig. 2A) and Poly I:C treated (fig. 2B) individual embryos. Expression values that were higher than the UCL for TNF-α of all embryos of CBA/J X Balb/C matings at day 8 of gestation were considered significantly increased.

Significantly, Increased TNF–α mRNA expression was observed in 2 out of 10 of the embryos in the untreated and 4 out of 9 in the Poly I:C treated females.

Simultaneous expression of TNF–α and iNOS mRNA

We have previously shown that increased infiltration of the decidua by maternal macrophages was associated with early fetal death. In addition we showed that the decidual macrophages at the implantation sites were primed *in vivo* as indicated by their expression of iNOS and responsiveness to LPS. In this study, the expression of the macrophage activation markers, iNOS and TNF-α, were determined in individual implantation sites of both untreated and Poly I:C treated pregnancies. Figure 3 shows a representative mating examined in this study. As shown in the Southern Blot (fig. 3A), embryo numbers 4, 8, and 9 showed an increased in iNOS mRNA and TNF–α mRNA expression. Furthermore, the number of embryos with a significant increase in both TNF–α and iNOS mRNA expression corresponds to the resorption rate observed in these matings. These studies at day 8 of gestation confirmed that the decidual macrophages were activated before apparent embryo damage since the same embryos showed significantly increased expression of two macrophage associated genes. We then extended our investigations to look at simultaneous expression

of iNOS and TNF–α mRNA expression in induced resorption. As expected, the results depicted in fig. 3B showed an increased incidence of simultaneous expression of TNF–α and iNOS mRNA in the putatively resorbing embryos further confirming our previous results. An occasional lack of concordance between iNOS and TNF-α expression was observed.

Quantitative Densitometric analysis of TNF–α and iNOS mRNA expression

In order to quantitate TNF–α and iNOS mRNA expression, we performed densitometric analysis on implantation sites taken from untreated and Poly I:C treated matings. The data was expressed as a ratio of either TNF–α or iNOS normalized with a housekeeping gene expression, G6PDH in this case. Figure 4a shows simultaneous expression of TNF–α and iNOS mRNA from untreated pregnancies. Note the existence of an increased expression in both TNF–α and iNOS mRNA in the same embryos (black and open bars respectively). Basal expression of TNF–α and iNOS mRNA was observed in the majority of untreated embryos. Figure 4B documents densitometric studies for Poly I:C treated matings. Embryos E2, E3, E4, and E5 showed increased expression of both TNF–α and iNOS mRNA at the same time. Embryos,

E6, E8, and E9 showed increased in iNOS mRNA expression, but basal TNF–α mRNA expression.

We have previously shown that embryos from normal low resorbing matings display similar cellular and molecular events to non-resorbing embryos of the spontaneously resorbing matings (4,8). These studies further showed a distinct bimodal distribution of resorbing and non-resorbing embryos. In this study, the data for iNOS and TNF-α mRNA of day 8 embryos was plotted as a frequency histogram. The expression of both iNOS and TNF-α mRNA showed a clear bimodal distribution (fig. 5). The day 8 embryos from low embryo loss matings showed relative iNOS and TNF-α expression of 0.0144 ± 0.038 and 0.026 ± 0.025 respectively, and the upper 95% confidence limit was 0.09 for iNOS and 0.08 for TNF-α expression. Using these values, expression of either iNOS or TNF-α mRNA were categorized as being normal or significantly increased. As shown in Table II, 32% of the embryos in the group of mice showed a spontaneous increase in iNOS mRNA expression corresponding to an expected resorption rate of 20%-30% (34). The percentage of embryos with increased iNOS expression in poly I:C treated pregnancies of 54% is also in accordance with the previously demonstrated incidence of induced resorption rate. These results show that an increase expression of iNOS mRNA is associated with early embryo loss. The results presented in table III indicates that

29.5 % of embryos showed spontaneously elevated expression of TNF–α mRNA. This incidence increased to 52 % when females were treated with Poly I:C. These studies demonstrated that increased TNF–α mRNA expression was associated with the elevated incidence of embryo resorption in normal and poly I:C treated CBA/J X DBA/2 matings.

Discussion:

The mechanisms by which allogeneic fetuses escape maternal immune surveillance are not fully understood. Suffice it to say that a large fraction of pregnancies terminate unsuccessfully (35,36). Exploration of this phenomenon has been focused on a mouse model in which CBA/J females mated with DBA/2 males, spontaneously abort 22% to 28% of their litters. The incidence of embryo loss is unrelated to litter size or paternal MHC expression (34). It was shown that TNF–α, which is produced by activated macrophages and NK cells induced fetal death if administered to pregnant females before day 10 of gestation. Recently, we showed that nitric oxide, a cytotoxic molecule produced by activated macrophages, is associated with early embryo loss. Since aminoguanidine, a preferential inhibitor of iNOS, successfully prevented embryo loss, it was apparent that NO was the ultimate effector molecules which caused the death of the growing fetus (8). Our previous

studies showed the expression of iNOS protein in cells with macrophage markers indicating that these decidual macrophages were the cellular source of NO production at the implantation sites (8). However, the low sensitivity of these assays prevented the accurate quantification of iNOS activity at day 8 of gestation. In this study, using RT-PCR and Southern blotting, we performed semi-quantitative analysis on iNOS and TNF-α mRNA expression at day 8 of gestation. The expression of iNOS and TNF-α mRNA as markers of macrophage activation was studied in implantation sites taken from both untreated and Poly I:C treated pregnancies. The results showed that 32% of embryos showed spontaneously increased iNOS mRNA expression and 29.5% showed increased TNF-α expression when implantation sites were taken from the untreated females. This percentage corresponded to the expected incidence of resorption for these mice (34). Similarly, 54% of the embryos showed elevated levels of iNOS mRNA expression and 52% showed increased TNF-α expression when mice were treated with Poly I:C. Again this percentage correlated with the incidence of resorption in Poly I:C treated mice. These studies indicated that iNOS and TNF-α expression was an excellent predictor of subsequent embryo resorption. We also showed that when the number of potentially resorbing embryos increased after Poly I:C treatment, increased iNOS and TNF-α mRNA expression was seen in a greater number of embryos. Hence, the

expression of iNOS and TNF-α mRNA at day 8 of gestation was an early event that preceded embryo damage normally apparent after day 10.

Lipopolysaccharide (LPS) and many other cytokines including IFN–γ are known to induce the expression of iNOS mRNA in macrophages. Further, treatment of pregnant mice with LPS and IFN–γ during early pregnancy induced fetal death confirming the association of iNOS expression with early embryo loss (37,38). Significantly, the accumulation and persistence of iNOS mRNA expression in macrophages depends on the continuous presence of external stimuli usually provided by IFN-γ (24, 25). Hence the expression of iNOS mRNA is not only an indication of macrophage activation, but also the presence of activating cytokines such as IFN-γ. The data further confirm that the activation of macrophages is an early event ultimately leading to early embryo loss.

Previous reports did not investigate the role of TNF-α in early embryo loss at the single embryo level (39,40). Studies have shown that exogenous TNF–α significantly increased embryo death. Furthermore, inhibitors of TNF–α production enhanced embryo survival (23). Previous reports have examined neither TNF–α mRNA expression at the single embryo level, nor the association with embryo resorption. Of note, several reports have demonstrated the presence of TNF–α mRNA in normal pregnancies (41,42). In addition, the expression of TNF–α mRNA

has been observed in cells that are physiological residents of uncomplicated pregnancies (43,44). In this study, we showed that 29.5% of embryos showed an increased TNF–α mRNA expression, while the remainder demonstrated barely detectable levels. This is in agreement with the above mentioned reports, documenting low levels of TNF-α in all normal embryos. This might indicate that at low concentration, TNF–α may have a morphogenetic role in embryonic development since we do not distinguish between expression by the placenta or the embryo itself (45). It was also noted that some embryos showed increased expression of only iNOS mRNA, with basal levels of TNF–α mRNA expression in some Poly I:C treated females. This may be due to the fact that the acute short term stimulation of cytokines stimulated by Poly I:C such type I interferon (46), could induce iNOS mRNA transcription without affecting TNF-α expression, by activating alternative transcription factors such as the interferon regulatory factor- element (IRF-E) (27). The direct induction of TNF–α mRNA expression by type I interferon has not been reported.

More importantly, the results of this study demonstrated that the embryos that showed an increase in iNOS mRNA expression are the same embryos that displayed an increase in TNF–α mRNA expression. The increased expression of both TNF–α and iNOS mRNA in the same embryos indicated that those embryos were infiltrated with activated

maternal effector cells. Though TNF–α can be produced by either NK cells or activated macrophages, our results do not conclusively indicate which cells are responsible for the production of TNF–α, but it is assumed that the macrophages are the most probable source. We have previously shown the expression of iNOS in decidual macrophages using double marker immunohistochemistry (8). Further, it is generally accepted that the expression of iNOS in macrophages is a strong indicator of macrophage activation and that TNF-α expression was necessary for NO production (8). On the other hand, neutrophils seem to be an unlikely source for TNF-α mRNA expression, since these cells were not notably present in the decidua at day 8 of gestation (47). Poly I:C induced NK cells cytotoxicity is independent of TNF–α production (48). On the other hand, we can not exclude the possibility that NK cells might be activated by IL-2 or IL-12. Both cytokines are known to induce TNF–α production by NK cells (31). In both cases, whether TNF–α was produced by NK cells or by macrophages, the results showed that TNF-α expression increased before embryo damage occurred. The results further showed that iNOS and TNF-α mRNA expression were correlated with embryo resorption. Activation of macrophages and expression of both iNOS and TNF–α mRNA could be a consequence of IFN–γ production by NK cells (49,50).

The results agree with our working hypothesis that in early embryo loss, activated decidual macrophages cause most of the embryo damage by producing nitric oxide and TNF-α. Prior to this event, NK cells may induce the activation of decidual macrophages by producing IFN-γ and TNF-α. However, it appears unlikely that NK cells directly mediate damage to the fetus, since NK cells directly isolated from implantation sites are poorly cytotoxic when assayed with Yac-1 target cells (Baines, unpublished data). During pregnancy, there was no observed difference in the frequency of decidual cells expressing the NK cytolytic molecule, perforin, between resorbing and non-resorbing embryos indicating a lack of association with early embryo loss (Haddad, unpublished data) and (51,52). However, it has been recently reported that there exist several phenotype subsets of NK-like cells in the uterus and other sites, and it is possible that these NK subsets may also differ functionally in terms of cytolytic activity and cytokine production (53). Finally, it is generally accepted that two signals are needed to induce the production of nitric oxide by macrophages (13). Therefore, TNF-α produced by NK cells, macrophages or other cells could indirectly act as a secondary signal to trigger the production of nitric oxide by primed decidual macrophages which leads to embryo death.

This study showed that increased iNOS and TNF–α mRNA expression at implantation sites are associated with early embryo loss. Indirectly, these results confirm the presence of activated macrophages at early implantation sites before the appearance embryo damage. This data lends further support to the hypothesis that NO and/or TNF-α are the most likely terminal effectors involved in fetal death, and that activated macrophages are the primary effector cells producing this outcome.

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References

1. Clark, D. A., M. R. McDermott, and M. R. Szewczuk. 1980. Impairment of host-versus-graft reaction in pregnant mice. II. Selective suppression of cytotoxic T-cell generation correlates with soluble suppressor activity and with successful allogeneic pregnancy. *Cell. Immunol. 52*:106.

2. de Fougerolles, A. R. and M. G. Baines. 1987. Modulation of the natural killer cell activity in pregnant mice alters the spontaneous abortion rate. *J. Reprod. Immunol. 11*:147.

3. Kinsky, R. G., G. Delage, N. Rosin, M. N. Thang, M. Hoffmann, and G. Chaouat. 1990. A murine model of NK cell mediated resorption. *Am. J. Reprod. Immunol. 23*:73.

4. Duclos, A. J., D. K. Pomerantz, and M. G. Baines. 1994. Relationship between decidual leukocyte infiltration and spontaneous abortion in a murine model of early fetal resorption. *Cell. Immunol. 159*:184.

5. Duclos, A. J., E. K. Haddad, and M. G. Baines. 1995. Presence of activated macrophages in a murine model of early embryo loss. *Am. J. Reprod. Immunol. 33*:354.

6. Baines, M. G., A. J. Duclos, A. R. Defougerolles, and R. L. Gendron. 1996. Immunological prevention of spontaneous early embryo resorption is mediated by non-specific immunostimulation. *Am. J. Reprod. Immunol. 35*:34.

7. Gendron, R. L. and M. G. Baines. 1988. Infiltrating decidual natural killer cells are associated with spontaneous abortion in mice. *Cell. Immunol. 113*:261.

8. Haddad, E. K., A. J. Duclos, and M. G. Baines. 1995. Early embryo loss is associated with local production of nitric oxide by decidual mononuclear cells. *J. Exp. Med. 182*:1143.

9. Bredt, D. S. and S. H. Snyder. 1990. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. USA 87*:682.

10. Stuehr, D. J., H. J. Cho, N. S. Kwon, M. F. Weise, and C. F. Nathan. 1991. Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc. Natl. Acad. Sci. USA 88*:7773.

11. Moncada, S., R. M. Palmer, and E. A. Higgs. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev. 43*:109.

12. Knowles, R. G. and S. Moncada. 1994. Nitric oxide synthases in mammals. *Biochem. J. 298*:249.

13. Ding, A. H., C. F. Nathan, and D. J. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol. 141*:2407.

14. Munoz-Fernandez, M. A., M. A. Fernandez, and M. Fresno. 1992. Synergism between tumor necrosis factor-alpha and interferon-gamma on macrophage activation for the killing of intracellular Trypanosoma cruzi through a nitric oxide-dependent mechanism. *Eur. J. Immunol. 22*:301.

15. Nathan, C. and Q. W. Xie. 1994. Nitric oxide synthases: roles, tolls, and controls. *Cell 78*:915.

16. Moncada, S. and A. Higgs. 1993. The L-arginine-nitric oxide pathway. *New Eng. J. Med. 329*:2002.

17. Griffiths, M. J., M. Messent, R. J. MacAllister, and T. W. Evans. 1993. Aminoguanidine selectively inhibits inducible nitric oxide systhase. *Br. J. Pharmacol 110*:963.

18. Hasan, K., B. J. Heesen, J. A. Corbett, M. L. McDaniel, K. Chang, W. Allison, B. H. Wolffenbuttel, J. R. Williamson, and R. G. Tilton. 1993. Inhibition of nitric oxide formation by guanidines. *Eur. J. Pharmacol. 249*:101.

19. Joly, G. A., M. Ayres, F. Chelly, and R. G. Kilbourn. 1994. Effects of NG-methyl-L-arginine, NG-nitro-L-arginine, and aminoguanidine on constitutive and inducible nitric oxide synthase in rat aorta. *Biochem. Biophys. Res. Comm. 199*:147.

20. Sidhu, R. S. and A. P. Bollon. 1993. Tumor necrosis factor activities and cancer therapy--a perspective. *Pharmacology & Therapeutics 57*:79.

21. Chaouat, G. 1994. Synergy of lipopolysaccharide and inflammatory cytokines in murine pregnancy: alloimmunization prevents abortion but does not affect the induction of preterm delivery. *Cell. Immunol. 157*:328.

22. Chaouat, G., E. Menu, D. A. Clark, M. Dy, M. Minkowski, and T. G. Wegmann. 1990. Control of fetal survival in CBA x DBA/2 mice by lymphokine therapy. *J. Reprod. Fert. 89*:447.

23. Gendron, R. L., F. P. Nestel, W. S. Lapp, and M. G. Baines. 1990. Lipopolysaccharide-induced fetal resorption in mice is associated with the intrauterine production of tumour necrosis factor-alpha. *J. Reprod. Fert. 90*:395.

24. Xie, Q. W. and C. F. Nathan. 1993. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon-gamma and bacterial lipopolysaccharide. *Transactions of the Association of American Physicians 106*:1.

25. Lorsbach, R. B., W. J. Murphy, C. J. Lowenstein, S. H. Snyder, and S. W. Russell. 1993. Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. Molecular basis for

the synergy between interferon-gamma and lipopolysaccharide. *J. Biol. Chem. 268*:1908.

26. Weisz, A., S. Oguchi, L. Cicatiello, and H. Esumi. 1994. Dual mechanism for the control of inducible-type NO synthase gene expression in macrophages during activation by interferon-gamma and bacterial lipopolysaccharide. Transcriptional and post-transcriptional regulation. *J. Biol. Chem. 269*:8324.

27. Martin, E., C. F. Nathan, and Q. W. Xie. 1994. Role of interferon regulatory factor 1 in induction of nitric oxide synthase. *J. Exp. Med. 180*:977.

28. Beutler, B., N. Kronchin, I. Milsark, C. Luedke, and A. Cerami. 1986. Contron of cachectin (tumor necrosis factor) synthesis: mechanisms of endotoxin resistance. *Science 232*:977.

29. Gifford, G. E. and M. L. Lohmann-Matthes. 1987. Gamma interferon priming of mouse and human macrophages for induction of tumor necrosis factor alpha production by bacterial lipopolysaccharide. *J. Nat. Cancer Inst. 78*:121.

30. Duclos, A. J., E. K. Haddad, L. E. Chalifour, and M. G. Baines. 1996. Embryo infiltration by maternal macrophages is associated with selective expression of proto-oncogenes in a murine model of spontaneous abortion. *Biol. Reprod. 54*:1088.

31. Degliantoni, G., B. Perussia, L. Mangoni, and G. Trinchieri. 1985. Inhibition of bone marrow colony formation by human natural killer cells and by natural killer cell-derived colony-inhibiting activity. *J. Exp. Med. 161*:1152.

32. Peters, P. M., J. R. Ortaldo, M. R. Shalaby, L. P. Svedersky, G. E. Nedwin, T. S. Bringman, P. E. Hass, B. B. Aggarwal, R. B. Berberman, D. V. Goeddel, and M. A. Palladino. 1986. NK-sensitive targets stimulate production of TNF–α but not TNF-b by highly purified human peripheral blood large granular lymphocytes. *J. Immunol. 137*:2592.

33. Chomczynski, P. and N. Sacchi. 1987. Single Step Method of RNA Isolation by Acid Guanidinium Thiocyanate Phenol Chloroform Extraction. *J. Anal. Biochem. 162*:156.

34. Baines, M. G., K. A. Billingsley, A. R. de Fougerolles, A. J. Duclos, H. J. Olney, D. K. Pomerantz, and R. L. Gendron. 1994. Evaluation of the role of exogenous pathogens on the incidence of embryo loss during early pregnancy in mice. *J. Reprod. Immunol. 26*:17.

35. Wilcox, A. J., C. R. Weinberg, J. F. O'Connor, D. D. Baird, J. P. Schlatterer, R. E. Canfield, E. G. Armstrong, and B. C. Nisula. 1988. Incidence of early loss of pregnancy. *New Eng. J. Med. 319*:189.

36. Roberts, R. M., C. E. Farin, and J. C. Cross. 1990. Trophoblast proteins and maternal recognition of pregnancy. *Oxford Rev. Reprod. Immunol. 12*:147.

37. Wegmann, T. G. 1990. The cytokine basis for cross-talk between the maternal immune and reproductive systems. *Curr. Opin. Immunol. 2*:765.

38. Tangri, S., T. G. Wegmann, H. Lin, and R. Raghupathy. 1994. Maternal anti-placental reactivity in natural, immunologically-mediated fetal resorptions. *J. Immunol. 152*:4903.

39. Yui, J., M. Garcia-Lloret, T. G. Wegmann, and L. J. Guilbert. 1994. Cytotoxicity of tumour necrosis factor-alpha and gamma-interferon against primary human placental trophoblasts. *Placenta 15*:819.

40. Tangri, S. and R. Raghupathy. 1993. Expression of cytokines in placentas of mice undergoing immunologically mediated spontaneous fetal resorptions. *Biol. Reprod. 49*:850.

41. Yelavarthi, K. K., H. L. Chen, Y. Yang, J. L. Fishback, and J. S. Hunt. 1991. Tumor necrosis factor alpha mRNA and protein in rat uterine and placental cells. *J. Immunol. 146*:3840.

42. Chen, H. L., Y. Yang, X. L. Hu, K. K. Yelavarthi, J. L. Fishback, and J. S. Hunt. 1991. Tumor necrosis factor alpha mRNA and protein are present in human placental and uterine cells at early and late stages of gestation. *Am. J. Pathol. 139*:327.

43. Garcia, F. U., H. L. Chen, Y. Yang, J. L. Pace, X. L. Hu, and J. S. Hunt. 1994. Tumor necrosis factor-alpha mRNA and protein in endometrial tumors: analysis by in situ hybridization and immunocytochemistry. *Hum. Pathol. 25*:1324.

44. Roby, K. F. and J. S. Hunt. 1994. Mouse endometrial tumor necrosis factor-alpha messenger ribonucleic acid and protein: localization and regulation by estradiol and progesterone. *Endocrinol. 135*:2780.

45. Gendron, R. L., F. P. Nestel, W. S. Lapp, and M. G. Baines. 1991. Expression of tumor necrosis factor alpha in the developing nervous system. *Internat. J. Neuroscience 60*:129.

46. Meurs, E., K. Chong, J. Galabru, N. S. Thomas, I. M. Kerr, B. R. Williams, and A. G. Hovanessian. 1990. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. *Cell 62*:379.

47. De, M., R. Choudhuri, and G.W. Wood. 1991. Determination of the number and distribution of macrophages, lymphocytes, and granulocytes in the mouse uterus from mating through implantation. *J. Leukocyte. Biol.* 50: 252.

48. Jewett, A. and B. Bonavida. 1995. Interferon-alpha activates cytotoxic function but inhibits interleukin-2-mediated proliferation and

tumor necrosis factor-alpha secretion by immature human natural killer cells. *J. Clin. Immunol. 15*:35.

49. Handa, K., R. Suzuki, H. Matsui, Y. Shimizu, and K. Kumagai. 1983. NK cells as a responder to IL-2: II. IL-2 induced IFN–γ production. *J. Immunol. 130*:988.

50. Weigent, D. A., G. J. Stanton, and H. M. Johnson. 1983. Interleukin 2 enhances natural killer cell activity through induction of gamma interferon. *Infect. Immun. 41*:992.

51. Parr, E. L., L. H. Young, M. B. Parr, and J. D. E. Young. 1990. Granulated metrial gland cells of pregnant mouse uterus are natural killer-like cells that contain perforin and serine esterases. *J. Immunol. 145*:2365.

52. Parr, E. L., H. L. Chen, M. B. Parr, and J. S. Hunt. 1995. Synthesis and granular localization of tumor necrosis factor-alpha in activated NK cells in the pregnant mouse uterus. *J. Reprod. Immunol. 28*:31.

53. Lachapelle, M.H., P. Miron, R. Hemmings, and D.C. Roy.1996. Endometrial T, B, and NK cells in patients with recrrent spontaneous abortion: Altered profile and pregnancy outcome. *J. Immunol. 156: 4027.*

Running Title:

Macrophage activation in early embryo loss

Figure Legends:

Figure 1. Expression of iNOS mRNA in individual embryos of normal and poly I:C injected CBA/J females mated with X DBA/2 males. Total RNA was isolated from individual implantation sites of untreated pregnant CBA/J females mated with DBA/2 males (A), or from Poly I:C injected (60 μg) CBA/J females (B). Total RNA was reversed transcribed and PCR amplified using primers specific for iNOS. The samples were separated on 1% agarose gel transferred to nylon membranes, and hybridized with ^{32}P labeled iNOS probe. The housekeeping gene, G6PDH was used as a control. The basal level of iNOS mRNA expression was determined from calculating the upper 95% confidence limit (UCL) of iNOS mRNA in embryos from low loss mating combinations at day 8 of gestation. Expression values that were higher that the UCL were considered significantly increased. Individual embryos were assigned numbers at the bottom of the figure.

Figure 2. Expression of TNF-α mRNA in individual embryos of normal and Poly I:C injected CBA/J females mated with DBA/2 males. For technical details see legend for figure 1. Expression values that were higher than the UCL for TNF-α mRNA expression at day 8 of gestation were considered significantly increased. Individual embryos were assigned numbers at the bottom of the figure.

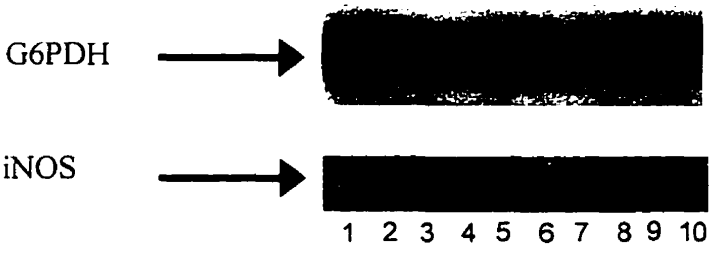
Figure 3. Simultaneous expression of TNF–α and iNOS mRNA in individual embryos of normal (3A) and Poly I:C treated (3B) CBA/J females mated with DBA/2 males. For technical details see legend for figure 1. Individual embryos were assigned numbers at the bottom of the figure.

Figure 4. Densitometric analysis of TNF–α and iNOS mRNA expression in CBA/J X DBA/2 matings. Simultaneous expression of TNF–α and iNOS mRNA in individual embryos of normal (4A) and Poly I:C induced (4B) CBA/J females mated with DBA/2 males. Open bars indicate iNOS expression and closed bars show TNF–α expression. Membranes were hybridized with ^{32}P labeled probes and subjected to densitometric analysis. The relative expressions of TNF–α and iNOS mRNA were normalized to the respective G6PDH signal of each embryo.

Figure 5. Frequency distribution of iNOS and TNF–α mRNA expression at day 8 of gestation. Relative expressions of TNF–α and iNOS mRNA were determined as described in the legend for fig. 1. Embryos that showed iNOS and TNF–α expression above the upper 95 % confidence limit were considered as potentially resorbing as indicated by the arrows.

Fig. 1 Expression of iNOS mRNA in Individual Embryos of Normal and Poly I:C Primed CBA/J

A)



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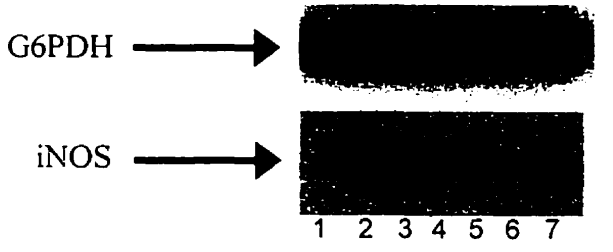
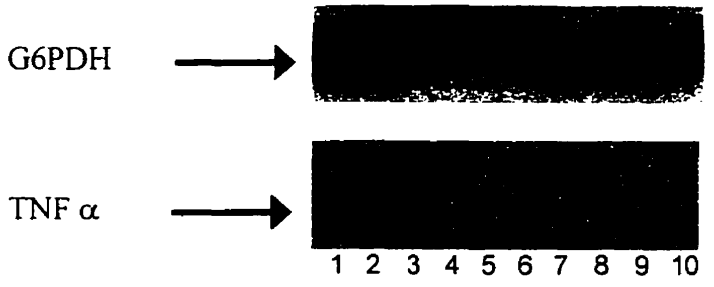


Fig 2. Expression of TNF-α mRNA in Individual Embryos of Normal and Poly I:C Injected CBA/J Females

A)



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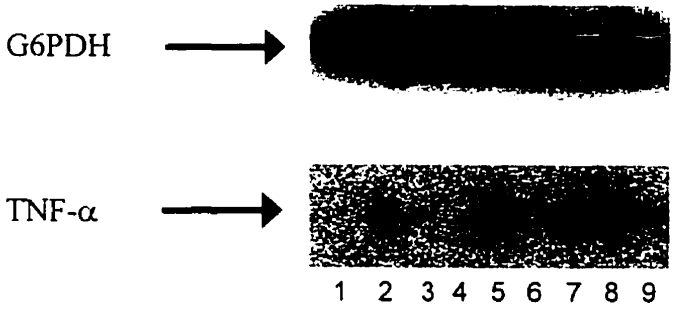
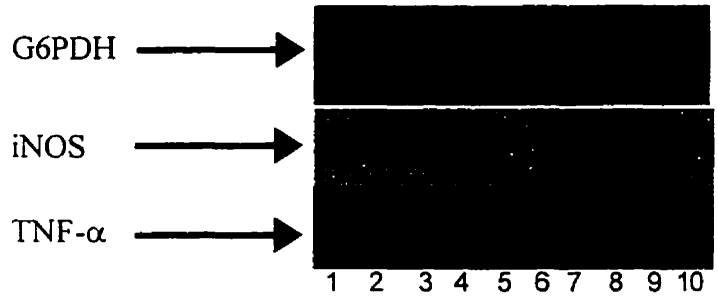


Fig 3. Simultaneous Expression of TNF-α and iNOS mRNA in Individual Embryos of Normal and Poly I:C Injected CBA/J

A)



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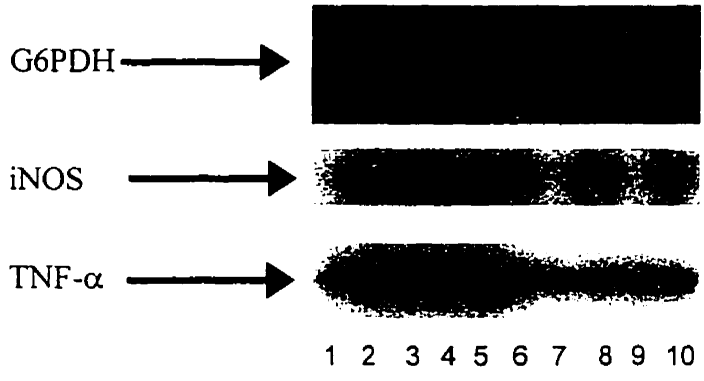


Fig. 4 Densitometric Analysis of Simultaneous Expression of TNF-α and iNOS mRNA in Normal and Induced Pregnancies

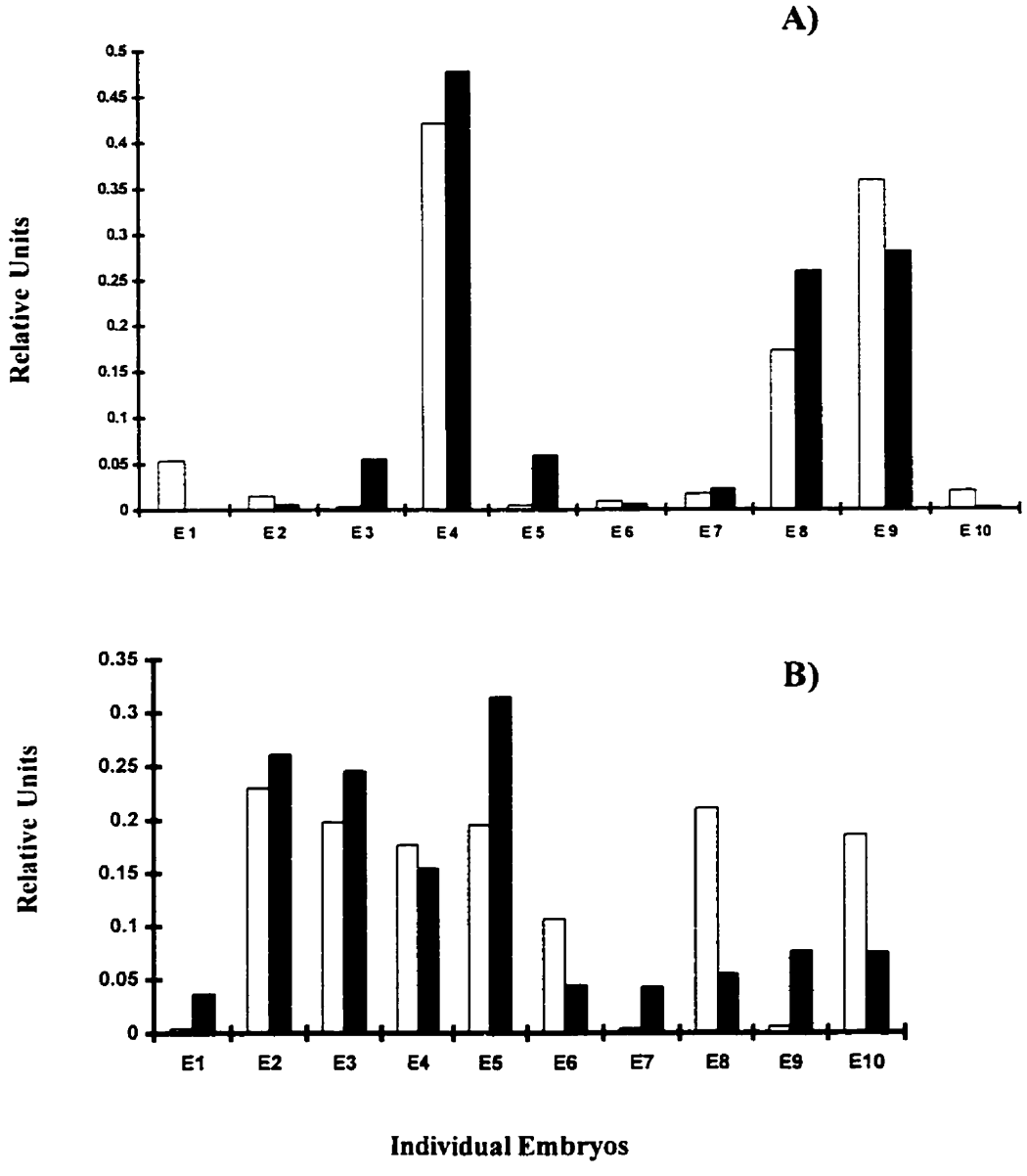


Fig. 5 The Frequency Distribution of iNOS and TNF-α mRNA at Day 8 of Gestation

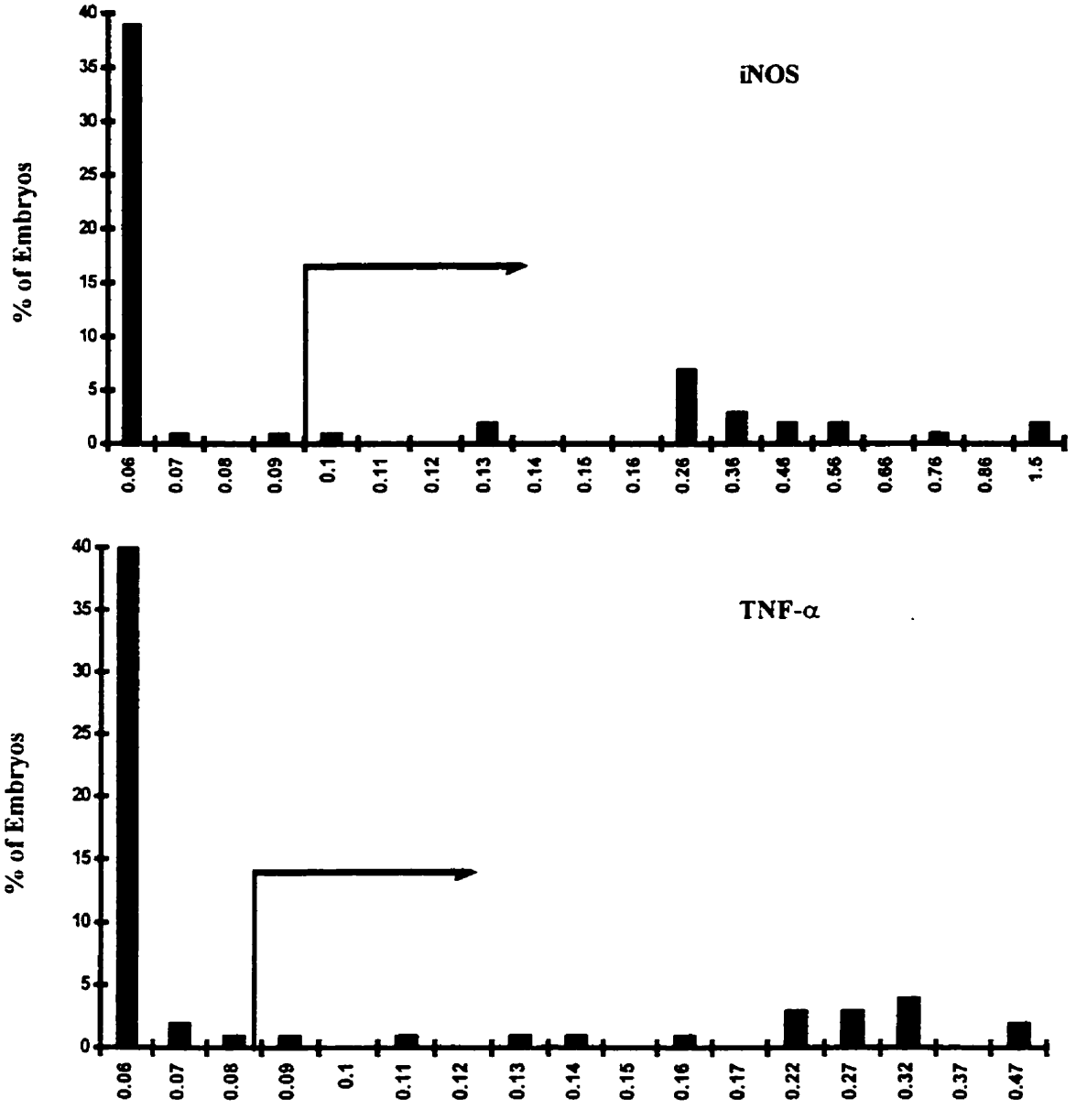


Table I. Primer and Probe for iNOS, TNF-α, and G6PDH Sequences

Specificities		Oligonucleotide Sequences	Expected PCR Product (bp)
iNOS	Sense	GAGCCTCGTGGCTTTGGGCTCCTC	486
	Antisense	GCGACGACGGTCTTTGAAGCCTTC	
	Probe	ACGTTCAGCACATCCTGCAAAAGCAGCTGC	
TNF-α	Sense	CCAGACCCTCACACTCAGAT	498
	Antisense	AACACCCATTCCCTTCACAG	
	Probe	CCAAGTGGAGGAGCAGCTGGAG	
G6PDH	Sense	CTAAACTCAGAAAACATCATGGC	111
	Antisense	GGAAGGTGGTTCGACTATGTG	
	Probe	GAGCAGGTGGCCCTGAGCCG	

Table II. Association of iNOS mRNA Expression and Early Embryo Loss in CBA/J X DBA/2 Matings at day 8 of gestation

Experimental groups	Number of Mice tested	Implantaion sites/mouse	Number of Embryos with increased* iNOS expression	Percent of Embryos with increased iNOS expression
Untreated Mice	6	10.1± 0.75	20	32%
Poly I:C Treated	5	7.8 ± 1.6	21	53.80%

* Expression of iNOS mRNA above the upper 95% confidence limit (> 0.09 relative units) established for all embryos from low loss matings of Balb/C X DBA/2 combination at day 8 of gestation was considered significant. Untreated or Poly I:C (60 μg) treated pregnant mice were sacrificed at day 8 of gestation Total RNA was isolated from individual embryos followed by RT-PCR and Southern blotting. Membranes were visualized and quantitated by phosphorimaging, and the relative expression of iNOS mRNA against a G6PDH control was determined.

Table III. Association of TNF-α mRNA Expression and Early Embryo Loss in CBA/J X DBA/2 Matings at day 8 of gestation

Experimental groups	Number of Mice tested	Implantation sites/mouse	Number of Embryos with increased* TNF-α expression	Percent of Embryos with increased TNF-α expression
Untreated Mice	6	10.1± 0.75	18	29.50%
Poly I:C Treated	5	7.8 ± 1.6	20	51.24%

* Expression of TNF-α mRNA above the upper 95% confidence limit (>0.08 relative units) established for all embryos from low loss matings of Balb/C X DBA/2 at day 8 of gestation was considered significant. Untreated or Poly I:C (60 μg) treated pregnant mice were sacrificed at day 8 of gestation. Total RNA was isolated from individual embryos followed by RT-PCR and Southern blotting. Membranes were visualized and quantitated by phosphorimaging, and the relative expression of TNF-α mRNA against a G6PDH control was determined.

Discussion and Future Directions

Early embryo loss is one of the most common complications of mammalian pregnancy that is responsible for more than 50% of embryo failures observed in early stages of gestation. Spontaneous and induced pregnancy failure in murine mating models proven to be invaluable in elucidating the mechanisms that mediate early embryo loss. Using these models, we have previously shown that the innate arm of the immune system, through the involvement of natural killer cells and macrophages, was associated in pregnancy failure. However, the mechanisms by which natural killer cells and macrophages mediate embryo abortion was not clearly understood. In this thesis, I have presented data that has conclusively illustrated the role of macrophages and natural killer cells in early embryo loss.

In chapter 2, we demonstrated that the production of nitric oxide by activated decidual macrophages was associated with early embryo loss. It was previously shown that two signals were needed to induce the production of nitric oxide by decidual macrophages. Therefore, cells from implantation sites of individual embryos were tested for the production of nitrite and nitrate with or without *in vitro* challenge with lipopolysaccharide, a source for the second signal. Furthermore, using

double immunostaining, decidual cells positive for both macrophage markers, Mac-1 and iNOS were demonstrated in implantation sites. The results strongly suggested a role for NO, produced by activated decidual macrophages, as an effector molecule in mediating early embryo loss and showed that the *in situ* activation of decidual macrophages was an early event preceding spontaneous abortion. In order to confirm that nitric oxide indeed was responsible for the embryo loss observed, we treated mice with aminoguanidine, a selective inhibitor for the production of nitric oxide. The results showed that mice treated with aminoguanidine showed a higher incidence of embryo survival than untreated controls. This indicated that nitric oxide not only was associated with embryo loss, but treatments that inhibited its production increased embryo survival.

In chapter 3, we investigated the role of IFN–γ as a first or primary signal in inducing macrophage activation and embryo resorption. In these studies, we studied the effect that the deficiency of IFN–γ genes and proteins had on early embryo loss. For this purpose, we used mice that were either engineered to lack the IFN–γ genes, or mice treated with anti-IFN–γ antibodies. The results showed that IFN–γ deficient mice were more resistant to LPS induced embryo loss than heterozygous control mice. This suggested that IFN–γ was needed for LPS induced

embryo resorption and that decidual macrophages in IFN–γ knockout mice were not primed and could not be activated when given LPS. We further studied the expression of IFN–γ and macrophage activation markers simultaneously in the same embryos. Our results showed that macrophage activation, subsequent nitric oxide production, and embryo loss were the results of local IFN–γ production.

It is very well established that Poly I:C induces embryo resorption when injected into a variety of mouse strains. Poly I:C are synthetic double stranded RNA that induces Type I interferon which causes NK cell activation. Therefore, we investigated the mechanism of poly I:C induced embryo resorption. The results indicated that IFN–γ deficient mice were significantly more resistant to Poly I:C induced abortion than wild type mice. This suggested that the mechanism of Poly I:C induced embryo abortion was, in great part, due to the production of IFN–γ by Poly I:C activated NK cells. The IFN–γ produced might prime decidual macrophages for nitric oxide production and embryo death.

Interleukin-12 (IL-12) has been first identified by its ability to activate NK cells. Furthermore, it has been shown that IL-12 played a fundamental role in regulating immune responses, particularly favoring Th1 cytokine production. Therefore, we studied whether the expression

of IFN–γ in some implantation sites was related to the presence of IL-12. Single embryos studies using RT-PCR have shown that IL-12 expression was detected in the same embryos that also showed TNF–α, IFN–γ, and iNOS mRNAs expression. This suggested that IL-12 could be involved in IFN–γ expression and subsequent nitric oxide production and embryo death.

In chapter 4, we studied the involvement of TNF–α in early embryo loss. The rationale behind these studies was to show whether TNF–α could provide IFN–γ primed macrophages with the secondary signal needed for the production of nitric oxide. The results showed that TNF–α was associated with spontaneous and induced early embryo loss. The results further showed that in the same embryos, the presence of TNF–α was associated with the presence of IFN–γ, and iNOS, indicating that TNF–α was associated with macrophage activation and nitric oxide production. Conversely, the results did not clearly exclude the possibility that TNF–α can induce direct damage to the embryo. In any of the cases, the mere presence of TNF–α would provide a secondary signal for the primed macrophages to trigger nitric oxide production.

The results presented in the present thesis demonstrated the cellular and molecular effectors associated with early embryo loss.

Future studies should address the nature of the initiating signals. In this regard, a number of hypothesis has been postulated. The mere presence of natural killer cells and T-cells with the γ/δ T-cell receptors at the implantation sites suggested that any or all of these cells could be involved. Besides, natural killer cells has been shown to be associated with early embryo loss. Furthermore, different factors known to induce natural killer cell and γ/δ T cells activation have been reported to be expressed at implantation sites. The conventional T-cells with α/β T cell receptors or B-cells are unlikely to be involved. First, these cells are rarely present early in gestation and second, the implantation sites are deficient in the classII MHC antigens needed to mediate recognition by α/β T cell receptors bearing cells.

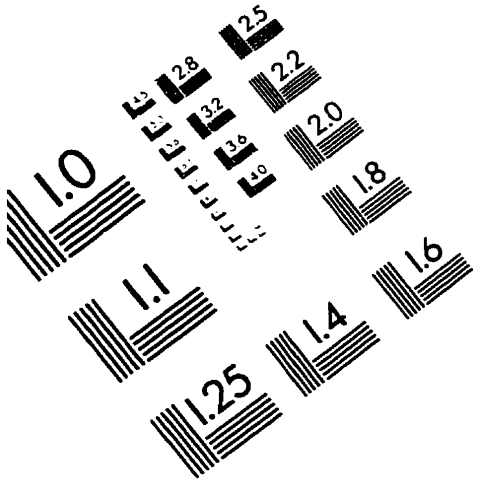
Finally, we envisage a model which incorporates the demonstrated cellular and molecular mediators of early embryo loss. The immunological initiating events may involve the activation of γ/δ T-cells but certainly involves NK cells. While in successful pregnancies the outcome of this activation is the production of TH2 cytokines such as IL-3, IL-4, and IL-10, in early embryo loss the production of TH1 cytokines such as IL-12, and IFN–γ becomes dominant.

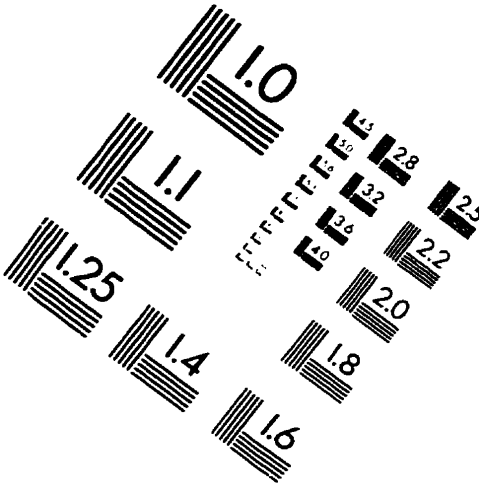
In early embryo loss, IL-12 produced by activated γ/δ T-cells induces activated NK cells to produce IFN–γ and TNF–α. IL-12 can also be

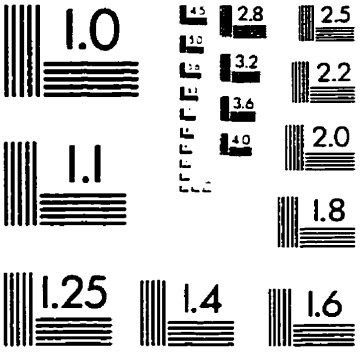
produced by decidual macrophages forming a positive feedback loop leading to IFN-γ production. The production of the latter provides the necessary signals for the activation of decidual macrophages, nitric oxide production, more TNF–α production, and killing of the growing embryo.

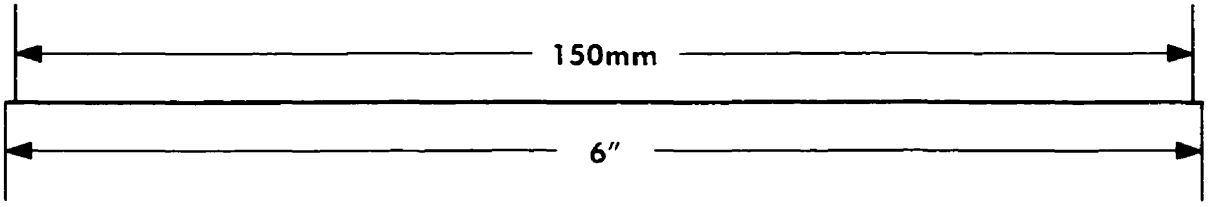
Further studies should address the nature of the initiating signal and further to determine the nature of the initial cytokines produced by γ/δ T-cells and NK cells. More importantly, future studies should determine the type of damage seen in the killed embryos. Our preliminary data suggests that the damage is inflicted to the placental cells which support the growth of the embryo by apoptosis or programmed cell death, and not the embryo itself.

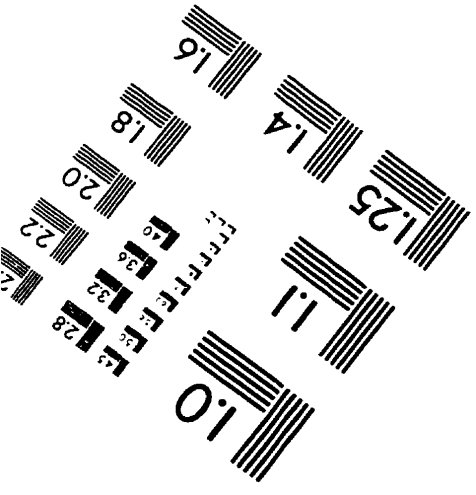
IMAGE EVALUATION TEST TARGET (QA-3)

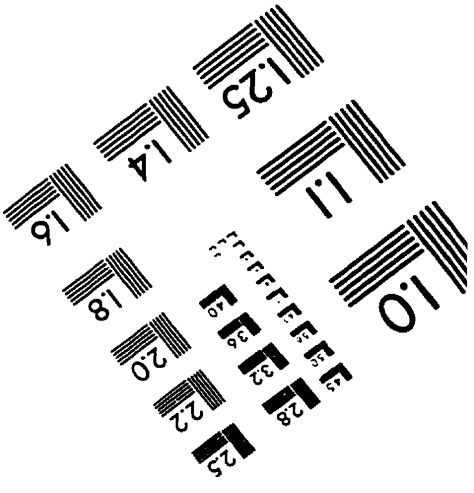












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